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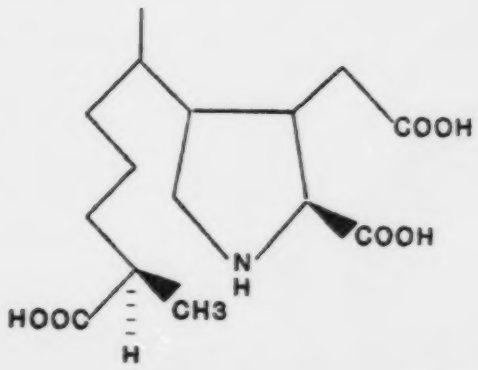
DOMOIC ACID TOXICITY

Ottawa, Ontario
11-12 April 1989



Health and Welfare Canada
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Canada

Proceedings of a Symposium

DOMOIC ACID TOXICITY

Ottawa, Ontario

11-12 April 1989

Organized by
the Laboratory Centre for Disease Control and the Food Directorate
Health Protection Branch
Department of National Health and Welfare

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1. Introduction

A.J. Liston

Ladies and gentlemen, it is a great pleasure to welcome you to Ottawa.

Allow me to say a few words about the purpose of this Symposium. As most of you already know, a mysterious illness occurred in Canada in November and December of 1987, which was soon traced to ingestion of mussels from the Atlantic coast. However, the clinical presentation of this new type of food-borne poisoning was so variable that for almost a week patients were treated as individual difficult-to-diagnose cases unrelated to each other. Only after a number of these cases appeared in Montreal within several days did the physicians start to suspect that ingestion of mussels may have been related to the disease. The Health Protection Branch was then alerted to that possibility and our testing has showed that some mussel extracts were toxic to laboratory animals. It also became clear that we were dealing with a substance never before encountered in human toxicology.

Regulatory measures developed jointly by the Department of National Health and Welfare (Health and Welfare Canada) and the Department of Fisheries and Oceans (Fisheries and Oceans) were introduced to protect the population from exposure to toxic seafood but, at that point, we did not know the nature of the toxin and our actions had to rely on general principles and common sense. A massive effort by dozens of closely cooperating scientists in the National Research Council, the Department of Fisheries and Oceans, and Health and Welfare Canada and in several universities and hospitals, subsequently identified the toxin as domoic acid. But, by then, the outbreak was well under control and no new cases of clinical poisoning were reported.

The emergency research efforts generated not only a wealth of new scientific information but also a long list of new, unanswered questions. A number of follow-up research projects addressed some of these questions and this Symposium was organized to review what we have learned in the 15 months since the beginning of the outbreak.

Ideally, we would like to have the scientific base and the required procedures to do 2 things: 1) be able to prevent any future outbreak of domoic acid poisoning; and 2) be able to diagnose and treat that poisoning if the safeguards fail. As you probably understand, the main function of the Health Protection Branch is to prevent a disease before it occurs. This goal requires not only constant vigilance, without which no preventive measures could be effective, but also sound regulatory policies based on scientific facts. The government has put into place safeguards to protect the Canadian population from an outbreak of seafood-borne domoic acid poisoning of the kind we experienced in 1987. But our regulatory position has to evolve in step with scientific progress because the safety of the consumer, which will always take precedence, is best guaranteed by increased knowledge.

We know now that domoic acid is constantly present in the marine environment and it is quite possible that in the future, with improved analytical methods, we will be able to detect traces of this toxic substance in other seafood products. We have to learn much more about the domoic acid toxicology to be able to decide responsibly on the safe and permissible level. We also have to learn much more about the fate of domoic acid in the marine environment to be able to predict the time and place of domoic acid accumulation so that ad hoc emergency measures can be replaced by orderly planning and effective management of the risks involved.

Obviously, the responsibility for fine-tuning these preventive policies will be shared by the regulatory components of the 2 departments involved: Fisheries and Oceans, and Health and Welfare Canada. However, the nature of the problem is so complex that the policies will have to rely heavily on the input of multidisciplinary bodies such as this gathering.

The main emphasis of this Symposium will, of course, be on the second general question: how to diagnose and treat domoic acid poisoning if it does occur. While we have every reason to believe that an outbreak of the 1987 type will be prevented, we cannot rule out the possibility that sporadic individual cases of domoic acid poisoning not necessarily related to commercial fishing will occur in Canada or in other countries due to an unforeseen combination of events in marine biology. In fact, we can neither confirm nor rule out the possibility that such individual and undiagnosed cases occurred in the past. The 1987 outbreak yielded a wealth of clinical experience which has to be summarized. We have here, in this room, many physicians and epidemiologists who have had firsthand experience from the outbreak. We have also invited a number of scientists who have made significant contributions to the original identification of the toxin as well as scientists involved in related theoretical research. Some and possibly most of the topics you will hear at this Symposium have already been presented individually at different meetings but I believe this is the first time we have had an opportunity to get a comprehensive picture.

The multidisciplinary composition of this audience also gives us an opportunity to address several specific questions which were selected as topics of the panel discussions during the next 2 days. The first one may appear to be a mere formality but I feel very strongly that this group should review once more the evidence that domoic acid was indeed the sole cause of the 1987 outbreak. I am told that a few scientists still have some doubt and it is necessary to make sure that no evidence or even alternate hypothesis was overlooked.

Another panel is planning to review the definition of the syndrome itself. We have to remember that in 1987 the clinicians were faced with a new, unknown disease for the first time and that the epidemiologists did not have the benefit of laboratory verification of the cases. It is quite possible that, in retrospect, some symptoms which appeared trivial and/or coincidental will gain significance and that laboratory

analysis will have to become part of the verification of diagnosis.

While it is my understanding that no fully effective antidote to domoic acid has been discovered yet, the panel on treatment will offer an opportunity to review the clinical effectiveness of treatment used by physicians involved in the 1987 outbreak and to compare this empirical experience with experimental findings of theoretical research.

Finally, we hope that the participants of this Symposium will identify those areas of research which have been overlooked and which appear to be quite essential for further progress in dealing with this syndrome in the near future.

I would like to wish you a very successful meeting.

2. *Chronology of the Toxic Mussels Outbreak*

E.C.D. Todd

On 22 November 1987, 2 persons in Moncton, New Brunswick, were hospitalized after suffering from gastroenteritis and mental confusion. Although there was no association between the cases, the symptoms seemed similar enough to question them about their food consumption, and it was found that they had both eaten mussels bought from 2 different retail stores. The next day mussels from the home of 1 patient were retrieved, and some more were obtained from the store from where he had purchased them. These were sent for testing to the Fisheries and Oceans mouse bioassay unit in Black's Harbour.

On 24 November reports of 2 cases were received by the Quebec Region of the Health Protection Branch from the Montreal Urban Community and the Quebec Community Health Department. These were 2 elderly men who had vomited and become confused with memory loss 4 to 5 hours after consuming mussels. On 25 November the Field Operations Directorate of the Health Protection Branch confirmed that mussels from either Prince Edward Island or Maine were linked to the illness and that Fisheries and Oceans was informed. Samples of mussels (2 from a restaurant, 1 from a home) from these 2 cases were packaged and were to be sent on 26 November to the Research Laboratories in Ottawa for mouse bioassay but a snowstorm delayed shipment. The next day they were driven by the Director of the Montreal Urban Community Laboratory to Ottawa for toxin testing. Although the symptoms (confusion and memory loss) of these 2 cases did not resemble paralytic shellfish poisoning (PSP) or any other known seafood toxicity, it was decided to inject extracts into mice to 1) eliminate (or confirm) the possibility of PSP and to 2) ascertain if any other toxic substance might be present. Extracts were prepared as for the standard PSP test and 2 mice were injected intraperitoneally for each sample. All the mice died between 15 and 25 minutes after being in an excitable state with a hind leg scratching reaction that had not been seen before. The results were relayed by telephone to the Montreal Urban Community and Fisheries and Oceans later in the day.

The same afternoon it was learned from Fisheries and Oceans that 1 of the samples analyzed in the same way at Black's Harbour had given a similar reaction. Also that day, the Health Protection Branch heard of 2 possible cases in Charlottetown, Prince Edward Island. It was later learned that 2 elderly persons had purchased mussels from a retail store on 25 November and had eaten them steamed and sprinkled with vinegar. Their dog had eaten a few at the same time and was the first to show distress by vomiting 3 times. The female also vomited a few hours later but the male was unable to do so and collapsed with prostration accompanied by head shaking. They had telephoned their physician for advice but they were ill for 2 days before visiting the hospital on 28 November. Samples of mussels from their home and from retail stores were collected and flown to Ottawa on the 28 and 29 November along with the samples from various leases on the Cardigan and Boughton Rivers. The case-associated mussels caused mouse deaths within 30 minutes preceded by typical scratching. The lease samples gave varying results, from no symptoms to scratching with or without deaths.

Distribution of mussels from Prince Edward Island was stopped on 29 November and when several more cases were reported from Montreal on 1 December a decision was taken on the same date to advise the public across Canada not to consume mussels from Prince Edward Island distributed after 1 November 1987, and mussels were taken off the shelves in retail stores and removed from restaurants. All provincial deputy ministers of health were notified by telex.

On 3 December an Analytical Working Group was organized to coordinate the various research efforts being started in government and other departments with input from Fisheries and Oceans, Health and Welfare Canada, the Department of the Environment (Environment Canada), the National Research Council, Prince Edward Island Fisheries, and the Atlantic Veterinary College. This group discussed progress every few days by conference calls originating from Fisheries and Oceans, Moncton, from 3 December 1987 until 12 January 1988, and examined mussels (and other shellfish), river mud, and plankton for pesticides, heavy metals, and pathogenic bacteria.

The first fatal case occurred on 9 December 1987, and another 4 deaths occurred 12 to 16 December and 9 March 1988, although the last fatality was not directly related to toxic mussel ingestion.

It was decided to test shellfish by bioassay not only from the eastern Prince Edward Island area but from all areas in the St. Lawrence estuary, Newfoundland, New Brunswick, and Nova Scotia coasts. In the next few days most samples did not show any toxicity, but mussels from the Magdalen Islands, Quebec, and oysters from 2 New Brunswick bays killed mice although not in the way that the Prince Edward Island mussels did. Nor was the reaction typical of PSP, i.e., paralysis-type death in 15 minutes. Although no illnesses had been associated with these toxic mussels or oysters, it was decided to prevent the sale of all live clams, oysters, mussels, and quahaugs from Atlantic Canada until further notice. Subsequently, it was found that the Magdalen Islands deaths were caused by low levels of PSP. Although this was suspected by the mouse bioassay analysts, it had to be confirmed by high-performance liquid chromatography (HPLC) analysis (Fisheries and Oceans, Mont-Joli, Quebec). As a result of this finding, all mice were examined for 18 to 24 hours instead of 15 minutes. The oyster toxicity problem took longer to solve because it had a lower priority (these oysters are not normally harvested in the winter) and was not investigated until the unknown toxin from Prince Edward Island was identified as domoic acid. The reason for mouse deaths was discovered to be high levels of zinc accumulated in the shellfish (800 ppm). The origin of zinc was not determined, but could have been either natural from

leached soils or industrial pollution. The high concentration of zinc kills mice many hours after injection but does not affect human beings; therefore, the oysters were later allowed to be sold.

In mid-December it became apparent that over 200 potential cases were associated with Prince Edward Island mussels with onset from 4 November to 5 December, although not all of these finally met the case definition. No other source of these illnesses was identified. On 18 December, the National Research Council announced that a toxin had been isolated from toxic mussels and determined it to be the excitatory amino acid, domoic acid (mol wt 311). By January 1988 an HPLC method for quantifying domoic acid in acid extracts of shellfish was developed and was immediately used for testing, although the mouse bioassay remained the definitive procedure for opening or closing areas. Environmental samples taken from various leases in rivers in eastern Prince Edward Island showed that domoic acid was present in mussels in late October (when harvesting began) until mid-January (when the levels had dropped to barely detectable figures, e.g., 1 to 4 ppm). Two leases in the Cardigan River supplied 98% of the toxic mussels eaten by cases.

At first, the origin of the domoic acid in the Cardigan area was postulated to be the red alga *Chondria baileyana*, a species found in Prince Edward Island waters. This is because the only known source of domoic acid until then was a Japanese seaweed, *Chondria armata*. However, even though a herbarium specimen of *C. baileyana* was found to contain domoic acid, the low mass of red seaweed in the estuaries would not be sufficient to account for the toxicity found in mussels. Other potential agents were dinoflagellates (e.g., *Prorocentrum*), pesticides, and heavy metals. These were eliminated because of lack of significant levels. Another source was plankton because samples collected in mid-December proved to be toxic to mice and were subsequently shown to contain domoic acid. The diatom *Nitzschia pungens* was the dominant organism in this plankton and pure culture work much later confirmed the source of domoic as being the *Nitzschia*.

Once the nature of the toxin was known and shellfish from the eastern seaboard tested, commercial harvesting was permitted in most areas by February. Mussels from eastern Prince Edward Island and the zinc-associated oysters from New Brunswick were released for sale in April.

Testing of shellfish by mouse bioassay for PSP with extended observation time and HPLC for domoic acid continued throughout the year. In the fall and winter of 1988, shellfish again became toxic, but to a lesser extent than in the previous year, and harvesting was prevented for shellfish with levels greater than 20 µg domoic acid/g. When the bloom of toxic *Nitzschia* in eastern Prince Edward Island had decreased early in 1989, shellfish were again available for consumption. No illnesses were documented from any of these mussels.

3. *Acute Manifestations of Domoic Acid Poisoning: Case Presentations*

J. Teitelbaum

In November 1987, 145 patients in Canada developed gastrointestinal and neurological symptoms following consumption of mussels from Prince Edward Island. The excitatory neurotoxin domoic acid was subsequently detected in the mussels associated with this mass intoxication.

In the following sections, 3 cases will be presented, illustrating certain aspects of this new clinical syndrome.

Case 1

The patient, an 84-year-old man, was followed for a pituitary adenoma diagnosed in 1985, rectal carcinoma resected in 1982 with no recurrence, and osteoarthritis. Medications included Indocid[R] and Entrophen[R] as needed. He did not smoke or abuse ethanol. Prior to mussel ingestion, the patient was entirely self-sufficient and still involved in the management of his real estate company. The patient ate a very large portion of Prince Edward Island mussels in his home at around 1800 hours on 19 November 1987. According to his family, about 1 hour later he began to have nausea, and protracted vomiting, lasting through the night. The patient was brought to hospital the next morning because of somnolence, confusion, and disorientation. On arrival, the patient was dehydrated, with a blood pressure of 110/80 and a pulse of 100. He was febrile, with a rectal temperature of 38.5°C. General examination revealed a right lower lobe pneumonia but was otherwise unremarkable. Neurological examination revealed marked disorientation in all 3 spheres, with confusion and intermittent delirium. Cranial nerves were normal. Motor examination showed normal bulk, with increased tone in the left upper extremity. Strength was mildly decreased in the left upper extremity with increased reflexes on the left. Plantar responses were flexor bilaterally.

Over the next few days the patient continued to deteriorate. He remained quite confused, with a high fever despite antibiotic therapy. By 10 days post-ingestion he reached his maximum disability. He was comatose but breathing spontaneously, with intact brainstem reflexes. Intubation was performed for airway protection from excessive bronchial secretions. Approximately 7 days post-ingestion, the patient developed focal motor seizures of the right arm, as well as generalized tonic clonic and absence-type convulsions. As well, he was noted to have facial myoclonus and uncontrolled chewing movements. Seizures were extremely difficult to control, being unresponsive to phenytoin and requiring large doses of intravenous diazepam and phenobarbital.

Routine laboratory data during this acute phase revealed a polymorphonuclear leukocytosis, increased urea with a mild increase in creatinine compatible with dehydration, and an increase in creatine kinase CK(MM) likely due to seizures. Lumbar puncture, done on 2 occasions, was completely normal. Electroencephalograms repeated many times during the acute illness showed diffuse slowing, with epileptic or epileptiform activity mainly in the left temporal area. Cranial computed tomography showed only diffuse atrophy, consistent with age. Electromyographic studies, performed 4 weeks post-ingestion, showed widespread denervation with normal conduction velocities. The patient slowly improved over the next 4 to 6 weeks. By 3 months post-ingestion, he was alert, with normal language, judgment and social skills. He was disoriented to place and time, with no recall of his acute illness. The patient was unable to retain any new information despite relatively normal retrograde memory function. Cranial nerves were intact. Tone and strength were normal, but there was marked wasting of the hands and feet. Reflexes were now decreased to 1+ except for the ankles, where they were unobtainable. Sensory examination was normal except for very mildly decreased vibration sense in the lower extremities.

Case 2

A 71-year-old male professor was treated for mild Parkinson's disease and peptic ulcers. His medications prior to ingestion included Artane[R] and cimetidine. There was no history of alcohol abuse. He ate Prince Edward Island mussels on the evening of 22 November 1987. He developed only mild nausea with no vomiting, diarrhea or abdominal pain. Twenty-four hours later, the patient was brought to his physician for mild somnolence, generalized weakness, and somewhat abnormal behavior. This intelligent man did not act normally, would forget what he was being told, and seemed unable to deal with the demands of his work. No precise diagnosis was made. For the next 3 days the patient still felt that he was much more fatigued than usual and his hands did not have their normal strength. As well, he noted decreased concentration, inability to remember faces, and difficulty with meetings that had just been planned, either forgetting the date of the meeting or what he was supposed to say. Four months later, the patient felt his memory was much improved and strength was subjectively back to normal. On physical examination 4 months post-ingestion, motor cortical functions and cranial nerves were intact. Strength was normal despite a mild decrease in bulk of the small muscles in the hands and feet. Reflexes were absent. Sensory examination revealed decreased vibration sense in the lower extremities.

Full biochemical work-up done 4 months post-ingestion was unremarkable. Cranial computed tomography, with and without contrast, was normal. An electroencephalogram showed mild generalized slowing of background activity. On neuro-psychological testing, his IQ was 130, language skills were normal, and the only abnormality was a decrease in visuospatial delayed recall. Electromyographic studies showed evidence of chronic and active denervation with mild diffuse axonal sensorimotor neuropathy. This could be

compatible with either motor neuron and dorsal root ganglion lesions, or diffuse axonal damage.

Case 3

A 69-year-old, right-handed man with well-controlled adult onset diabetes, hypertension, and atherosclerosis, ingested Prince Edward Island mussels on 20 November 1987. His medications prior to ingestion included Euglucon®, methyldopa and enteric aspirin. His alcohol intake was moderate, and he smoked 25 cigarettes per day. Three hours after ingestion, the patient developed severe nausea and vomiting. Twenty hours post-intoxication, he was unresponsive. A few hours after that, the patient developed gastrointestinal bleeding and went into shock due not only to hypovolemia, but also to a massive vaso-dilatation which spontaneously resolved 10 hours later. While comatose, the patient had retained intact brainstem reflexes. Ten days post-ingestion, the patient developed a novel motor syndrome. While verbally unresponsive, he was found to have a paresis of his right side with ipsilateral increase in tone and reflexes. This lasted 24 to 36 hours, resolved, re-appeared on the left side for another 24 to 36 hours, and then resolved completely. At the same time, he developed complete external ophthalmoplegia, with no caloric or oculocephalic reflexes. Despite this, the patient could blink, swallow, and breathe. Over the next 10 days his eye movements became disconjugate and then slowly returned to normal. No seizures were noted. Over the next 12 weeks the patient improved, slowly regained consciousness and became alert. His language was normal and he was noted at this time to have a severe problem with recent memory and concentration. He had some retrograde amnesia but the most striking finding was an inability to retain new information. His motor examination 12 weeks post-ingestion revealed decreased muscle bulk in his hands and his feet, but his strength seemed to be normal as were his reflexes. Sensory examination revealed decreased sensation to light touch and pain over the feet and hands, with a decrease in vibration in the lower extremities. Position sense was normal. Complete blood count and biochemical profile were performed on the day of arrival in hospital. Polymorphonuclear leukocyte count was elevated, as were urea and creatinine. CK(MM) was mildly elevated as well. Computed cranial tomography, with and without contrast, was normal. Cerebrospinal fluid analysis was unremarkable. He had multiple electroencephalograms done. At 5 days after ingestion he had some generalized slowing of activity more marked on the left side than on the right. Two weeks later, the electroencephalogram (EEG) pattern was compatible with a metabolic encephalopathy, with no epileptiform activity noted. Four months later, the EEG had improved, with mild to moderate generalized disturbance of background activity. Electromyographic studies performed 4 weeks after ingestion revealed marked active denervation in proximal and distal muscle groups with normal conduction velocities. Four months later, denervation was improved, and there was evidence of mild sensorimotor axonal neuropathy.

Discussion

As these 3 cases demonstrate, acute manifestations of domoic acid intoxication are variable. The common features are those of nausea and vomiting, followed by varying degrees of confusion, disorientation, changes in level of consciousness, and seizures in some cases. Motor abnormalities (hemiparesis, ophthalmoplegia) were seen transiently in 2 cases during the acute phase. The residual memory abnormality and sensorimotor neuropathy/axonopathy are rather unique late manifestations that have now been studied in a larger population of affected patients. Hopefully, recognition of the intoxication will be made easier from the data we report.

4. *Amnesic Shellfish Poisoning: A New Clinical Syndrome Due to Domoic Acid*

T.M. Perl, L. Bédard, T. Kosatsky, J.C. Hockin, E.C.D. Todd, L.A. McNutt, and R.S. Remis

Introduction

In late November 1987 reports of severely ill individuals with acutely developed confusion, profound memory loss, and disorientation were received. The 4 index cases had ingested mussels cultured in eastern Prince Edward Island within 24 hours of symptoms onset. Although these manifestations were inconsistent with paralytic shellfish poisoning (PSP), to eliminate this possibility, mussels recovered from the implicated meals were sent for standard PSP toxin analysis by the mouse bioassay. Additional cases throughout Canada were rapidly identified by an active search. The suspected mussels were recalled by Canadian authorities and on 1 December 1987 a public warning against eating mussels from eastern Canada was issued.

Methods

Case finding and follow-up

A case of mussel-related illness was defined as any person who consumed mussels from PEI on or after 1 November 1987, and who developed one or more of the following gastrointestinal symptoms within 24 hours: vomiting, diarrhea, or abdominal cramps; or at least one of the following neurological symptoms or signs within 48 hours: confusion, memory loss, disorientation, or other major objective signs such as seizures, coma, and cranial nerve palsies. Cases were identified by local, provincial, and national departments of health. After receipt of preliminary reports, questionnaires were administered to most cases by telephone. Additional clinical data was obtained by reviewing hospital charts, discharge summaries, and postmortem examinations.

Laboratory studies

Where possible, mussels associated with illness were obtained from restaurants, homes, and retailers. Serum or plasma specimens collected from 17 cases in Quebec were tested for domoic acid.

Tracing of mussel origin

We attempted to determine the source of mussels consumed by cases. The November bills of sale from the distributors to restaurants and retailers and from mussel producers to distributors were reviewed and compared. The source of mussels was accepted as Prince Edward Island if the bills of sale indicated that the distributor had received mussels from Prince Edward Island within 14 days of sale to the restaurant or retailer.

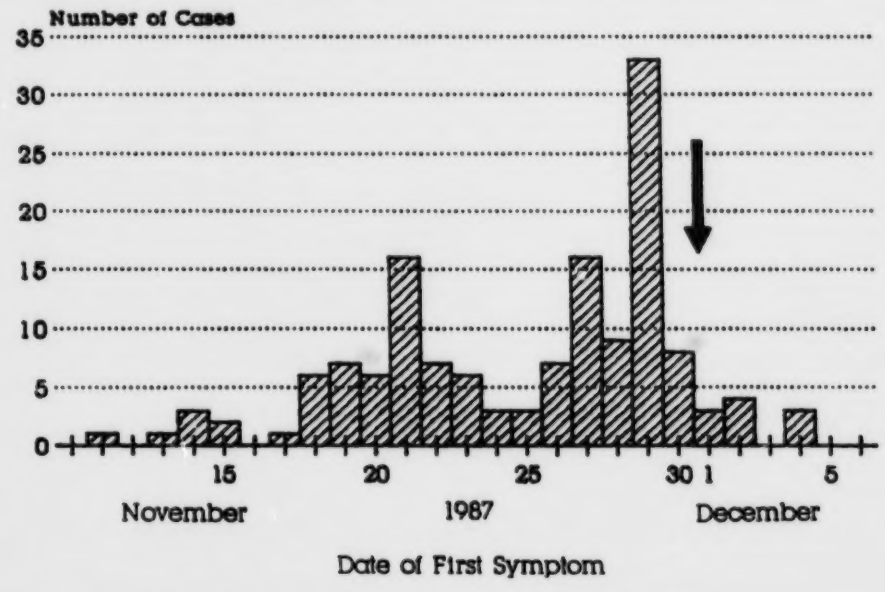
Results

Description of cases

We collected 229 preliminary reports of illness related to consumption of mussels in Canada with onsets from 4 November to 4 December 1987. Of these reports, 107 cases were identified based on data collected from questionnaires. An additional 38 probable cases were identified using information gathered from other sources including hospital chart review and follow-up information. No cases of illness were confirmed outside of Canada (Figure 1).

The data presented here are based on 145 confirmed or probable cases. Men and women were affected in equal numbers. Eighty per cent of the cases were over the age of 40 and almost 60% were between the ages of 40 and 60. The geographic distribution and attack rates by province are presented in Table 1.

Figure 1
Number of Canadian Cases of Mussel-Associated Intoxication by Date of Symptom Onset
November - December 1987



Arrow: date of withdrawal of mussels from the market and the warning to the public

The time from ingestion to the onset of first symptom varied from 15 min to 38 h (median 6 h). Onset of neurologic symptom following consumption of mussels ranged from 2 to 58 h with a median of 16 h. Illness was manifested by both gastrointestinal and neurologic symptoms (Table 2) which ranged from mild to severe enough to require hospitalization. At least one objective neurologic symptom was present in 37/145 (26%) of the cases.

Eighteen per cent (26/145) of the cases were hospitalized, 50% (13/26) in intensive care units (ICU). The development of neurologic illness and severe illness requiring ICU care was associated with age.

Four deaths occurred within 24 days of hospitalization. One severely affected case died of a myocardial infarction 3 months after hospital discharge.

Receiving treatment in an intensive care unit and death were directly related to age; 11 of 13 patients in the ICU and all deaths were in individuals over 68 years of age. The 2 younger patients had chronic renal disease requiring dialysis.

Laboratory analysis

Domoic acid concentrations in samples of mussels associated with illness were available for 10 cases and varied from 31 to 128 mg/100 g. Quantitative analysis of domoic acid from the implicated mussels that were recovered suggested that increasing levels of domoic acid in mussel samples were associated with increasing severity of illness.

Tracing of mussel origin

Forty-eight different restaurants and 20 households were involved during the time period of interest. Seventy-two per cent of the individuals ate in restaurants. Nineteen distributors were sources of mussels consumed in these restaurants and households.

In November 1987 approximately 75,000 kg of mussels were sold by the 2 PEI producer-cooperatives. Almost half of these went to the province of Quebec, the remainder to the rest of Canada and to the United States. One hundred and forty of the 145 cases (97%) ate mussels traced to leases in the Cardigan River, all of which were located within 500 m of each other. The 5 other cases of mild gastrointestinal illness were traced to 2 other river estuaries. In 3 cases the mussels originated from the Boughton River and two cases from the Brudenell River.

Following recall of all implicated mussels from restaurants and distributors, no new cases occurred.

Table 1.
Attack Rates by Region for Mussels from Implicated PEI River Estuary, November 1987

Region	No. of Cases	Kg of mussels shipped	Cases per 1,000 servings (750 g each)
United States	0	7,568	0.0
Alberta, Manitoba, Saskatchewan	0	5,705	0.0
Nova Scotia, Newfoundland	0	150	0.0
New Brunswick	1	1,750	0.4
Ontario	15	18,273	0.6
British Columbia	7	3,818	1.3
Quebec	109	41,341	1.9
Prince Edward Island, Canada	13	4,145	2.3
Total	**145**	**75,182**	**1.4**

Table 2.
Symptoms Associated with Mussel Associated Illness for 145 Canadian Cases, November - December 1987

	Frequency[1]	% of Cases
Gastrointestinal symptoms	**135/145**	**93**
Nausea[1]	116/14	80
Vomiting	94/145	64
Abdominal cramps	59/145	41
Diarrhea	59/145	41
Anorexia[1,2]	47/113	42
Neurological symptoms[2]	**37/145**	**26**
Headache	64/145	34
Loss of balance/dizziness[1]	48/145	33
Memory loss	37/145	26

[1] Not accepted for case definition.

[2] Includes only memory loss, confusion, disorientation, or other objective sign; does not include loss of balance, diziness.
Not all symptoms were available for all cases but a conservative estimate was used and, if the response was unknown, it was assumed not present.

5. *Neurological Sequelae of Domoic Acid Intoxication*

J. Teitelbaum, R. J. Zatorre, S. Carpenter, D. Gendron, and N.R. Cashman

Introduction

From 11 November to 4 December 1987, an outbreak of illness associated with the ingestion of mussels was observed. Field epidemiologists traced all responsible mussels to 2 mussel farms in a single river estuary in Prince Edward Island [1]. The clinical presentation in these patients was unlike any previously reported shellfish-related disease, and seemed to represent a new clinical syndrome.

According to T. Perl et al. (see Presentation 4), 145 patients are thought to have been affected, 70% from the province of Quebec. Neurological symptoms were present in one third of cases, and 4 patients died as a result of ingestion. Acute neurologic manifestations included headache, disequilibrium, confusion, an altered state of arousal ranging from agitation to coma, and convulsions in some. General manifestations included nausea and vomiting, with or without diarrhea, and abdominal pain. Investigators from the National Research Council identified domoic acid, a glutamate analog, in extracts of mussels, and suggested that it was the toxin responsible for the syndrome[2].

Glutamate is an excitatory neurotransmitter that has varying affinity to 3 types of receptors: quisqualate (Quis), N-Methyl-D-Aspartate (NMDA) and kainate (KA). Domoic acid (DA) binds to the kainate receptor 3 times more readily than KA and is 20 times more powerful. In the presence of glutamate, DA can cause massive cell depolarisation with subsequent increase in intracellular Ca++ and resultant cell dysfunction or death. After following 3 patients hospitalized in our institution for mussel ingestion, Dr. Neil Cashman and I noted that, although the patients recuperated well, they were left with some striking mental status abnormalities. In order to assess neurological sequelae in these and other patients having ingested toxic mussels, we studied the clinical evolution of 14 patients over a 12-month period following ingestion.

Materials and Methods

Clinical protocol

Cases identified by T. Perl and co-workers at Health and Welfare Canada were contacted by telephone and asked to participate in our study. Fourteen patients initially agreed. Patients and family were interviewed using a standardized-item questionnaire, with emphasis on initial neurological symptoms and their progression over the ensuing months. Past medical history and prior level of function were assessed. Charts were reviewed for all patients hospitalized during the acute illness. Complete neurological examinations were performed on 3 patients during acute illness, and 4, 8, and 12 months later in all cases.

Laboratory data obtained during the acute phase of illness were reviewed. Four to 6 months post-ingestion complete blood count, biochemical profile, electroencephalogram, and electromyographic studies were performed on all patients. Cranial computed tomograpy (CT) was obtained on those patients who had not undergone this procedure initially, or on whom the previous CT was abnormal. Five patients had magnetic resonance imaging of the brain as part of a Mental Research Institute positron emission tomography (MRI-PET) protocol[3]. Electromyographic and electroencephalographic studies were again repeated 8 to 10 months after the acute event.

Mental status abnormalities were a prominent feature of this syndrome. All patients underwent extensive standardized neurological testing evaluating IQ, language, memory, and frontal lobe functions. Verbal and performance IQ were calculated using the revised Wechsler adult intelligence scale [4]. Verbal comprehension and fluency were evaluated using De Renzi's test [5] and Thurstone and Thurstone's word fluency test [6]. Object naming was evaluated using the Wingfield test [7]. For frontal lobe functions, the Wisconsin card sorting test was used [8].

Visuospatial memory was tested using the Rey Complex figure [9] with immediate copy and 45-min delayed recall. In order to assess the retention of verbal material, the Wechsler memory scale was modified by the addition of a 90-min delayed recall.

Histological Preparations

The formalin fixed brains of the 4 patients who died 7, 12, 25, and 98 days post-toxic mussel ingestion were obtained. Their clinical manifestations are not amongst those of the 14 that we follow regularly. Representative sections were taken from all cortical areas including insula. Both hippocampi and amygdala were examined, as well as representative sections of basal forebrain, thalamus, hypothalamus, basal ganglia, brainstem, and cerebellum. Paraffin sections were stained with hematoxylin-eosin, Holzen, periodic acid Schiff, Bielschowsky's stain and Luxop Fast Blue.

Results

General medical manifestations

In our group of 14 patients, all but one presented with nausea and vomiting 1.5 to 24 h post-ingestion. Twelve of these 13 also had abdominal pain while only 3 had diarrhea. Bronchial secretions were a major problem in 7 patients, and required intubation in 6, all of whom became unresponsive. After reaching a peak in their deficits, patients stabilized and began to slowly improve. It took from 24 h to 12 weeks for these patients to become alert and able to respond appropriately to their environment. This interval was longest in those who were most severely affected post-ingestion. Six patients were hyperthermic with an infectious focus in 4. All 8 patients who were hospitalized post-ingestion showed some degree of hemodynamic instability and brady/tachyarrhythmia, but only 3 of our patients went into shock with blood pressure >90/60 (1, 8, 12). In all 3 cases, blood pressure was corrected in less than 6 hours. The cause of the decreased blood pressure seemed to be a transient decrease in vascular resistance.

Four months later, all these manifestations have cleared, and the only non-neurological problems remaining are those that were present prior to ingestion.

Neuropsychological Profile

All our patients showed some degree of confusion 4 to 72 h after ingestion. Level of consciousness was affected in 12. Six patients presented agitation or somnolence, while 6 others became comatose. Time to maximal deficit varied from 4 h in the least affected to 72 h in those who became unresponsive.

Formal psychological testing was performed 4 to 6 months after intoxication. Most subjects did not show evidence of global deterioration, and IQ scores were in keeping with premorbid levels of intellect. All patients performed well on tests of language and frontal lobe function. Except for one patient who had a normal neuropsychological profile (case 4, 44-year-old female), and one patient with global impairment (case 7, 87-year-old male), all remaining cases displayed a disproportionate disturbance in memory as compared to other cognitive functions. Memory deficits varied in severity, affecting mainly delayed recall of verbal and visuospatial material. Testing is evaluated in more detail by Dr. Zatorre [10].

Seizures and Myoclonus

According to T. Perl et al., 6.8% of all persons affected had convulsions. In our group of 14 patients, 5 had seizures during the acute phase of intoxication. Seizure types included focal motor, generalized tonic-clonic, and partial complex. Four of the 5 patients had chewing movements and facial myoclonus. The fifth had abnormal posturing of the left arm. Seizures were relatively resistant to Dilantin^{R} and required high doses of intravenous Valium^{R} and phenobarbital for control. By 4 months post-ingestion, seizures were no longer present.

Electroencephalographic studies were obtained acutely in only 7 patients. All had moderate to severe generalized disturbance of activity. Only one patient had epileptiform activity on EEG. Four months later, 3 patients had normal findings and all had improved.

Motor Abnormalities

During the acute phase post-ingestion, all patients complained of generalized weakness and unsteadiness. Only 4, however, had objective findings.

Two patients developed a virtually identical syndrome during their acute illness, consisting of alternating hemiparesis and ophthalmoplegia. Hemiparesis, with ipsilateral increased tone and hyperreflexia, resolved after 24 to 36 h, followed by transient occurrence on the opposite side. Eye movement abnormalities were initially characterized by complete ophthalmoplegia, with absent oculocephalic or oculovestibular reflexes despite normal blink responses and corneal reflexes. This slowly resolved and, by 10 days after onset, extra-ocular movements were normal.

Ophthalmoparesis alone was present acutely in 2 other patients in whom abduction defects suggested bilateral 6th nerve palsies. Diplopia resolved in 24 h in 1 patient, but required 12 weeks to normalize in the other.

Cranial computerized tomography was normal in 5 patients studied acutely, and in 5 other patients at 4 months.

Four months post-ingestion, 9 patients complained of mild weakness, accompanied by muscle cramps in 2, and fasciculations in 3.

In 2 cases, serial electromyographic studies were performed 2, 6, and 16 weeks post-ingestion. Concentric needle examinations revealed acute denervative changes, while motor amplitudes were decreased by 10%-20%, and nerve conduction velocities were almost normal (decrease of 0%-10%). Four to 6 months post-ingestion, 11 patients were tested. Two patients had normal studies. Nine had abnormal findings on concentric needle examination. Six of the 9, 3 of whom were diabetic, had 10%-30% decrease in amplitude of motor unit potentials with only 0%-10% decrease in motor nerve conduction velocity. Sensory studies were even less affected, with essentially normal velocity and an amplitude decrease of 0%-10%.

Results suggest either a primary anterior horn cell and dorsal root ganglion pathology or a diffuse axonopathy.

Histological Studies

These have been reported in detail by Dr. S. Carpenter [11]. Briefly, the areas most severely affected in the 4 necropsied cases were the hippocampus and amygdaloid nucleus. Thalamic damage was prominent in cases 2 and 3 (particularly in the medial dorsal nucleus) but minimal in cases 1 and 4. Damage to the insular and subfrontal cortex (especially the 6th cortical layer) was present in the first 3 cases and absent in case 4. All showed lesions in the claustrum, secondary olfactory areas, the septal area and the nucleus accumbens septi. There was necrosis or loss, depending on the time interval, of all pyramidal neurons, except for field H2, where there was moderate sparing. In case 2, the astrocytic reaction was more advanced in H3, 4, and 5 than in H1. In cases 3 and 4, H1 showed some cavitation. Dentate cells were involved in all except case 2.

Discussion

Are the signs and symptoms we describe truly related to DA intoxication? The evidence is indirect but strong. DA was not found in serum, and samples of urine and feces for DA analysis were not obtained from the acutely ill patients. Nevertheless, the relationship of the acute illness to ingestion of mussels in the previous 24 h was consistent. The source of the mussels in all cases was traced back to 2 leases in 1 estuary in Prince Edward Island, and mussels distributed from these 2 leases were found to contain DA and no other toxin. As well, the uniform topography of lesions in the brains of the 4 patients who died corresponds well with what has been reported in experimental animals after intoxication with kainic acid, a DA analog.

The majority of patients in this sample showed clear evidence of selective memory loss, with preserved general intellectual ability and intact language function. The memory deficit shown by these patients resembles closely the amnesia originally described by Scoville and Milner in 1958 [12] in a patient who had undergone bilateral excision of the amygdala and hippocampus. The pathological findings of bilateral hippocampal lesions in the 4 patients who died of mussel intoxication support this hypothesis. The lesions demonstrated in the hippocampi cannot be attributed to anoxic damage, as only 3 of 14 patients are reported to have had a

blood pressure below 90/60 mmHg and there was no evidence of ischemia on cranial computerized tomography.

Domoic acid, an excitatory amino acid found in the seaweed *Chondria armata* [13], and now isolated from *Nitzschia pungens* [14] in Prince Edward Island waters, is thought to be the agent responsible for this syndrome. The seaweed has been used for hundreds of years in Japan as an ascaricide and insecticide [15]. Domoate is structurally related to the excitatory amino acid kainate, but has a 3-fold higher affinity for the kainate receptor in the central nervous system (CNS) [16-19]. There are striking similarities between the abnormalities produced by domoic and kainic acid on experimental animals and the effects of mussel ingestion in our patients.

Acutely, systemic or intra-striatal injection of kainic acid in rats produces wet-dog shakes, masticatory movements, rearing, forelimb clonus, and limbic seizures (confirmed by depth electrodes) which are relatively resistant to phenytoin and more amenable to barbiturate and benzodiazepine therapy [20-22].

Systemic or intra-striatal injection of kainic acid in rats produces chronic widespread neuronal degeneration in the olfactory cortex, amygdaloid complex, hippocampus, and related parts of the thalamus and cortex. Cerebellum, brainstem and spinal cord are reportedly unaffected [20-24]. Hippocampal lesions are maximal early in the pyramidal cells in subfields CA3 and CA4.

Domoate and kainate are known to affect spinal cord neurons in several animal models. Electromyogram (EMG) evidence of denervation in our patients may be explained by a direct cytotoxic action on anterior horn cells and dorsal root ganglion cells. Alternatively, domoate might cause a diffuse axonopathy, but no mechanism is known by which it could do so.

The epidemic whose sequelae we have reported appears to have been a unique event, which should not recur. The levels of domoic acid are now closely monitored in the estuaries of Prince Edward Island where mussels are cultured. The possibility remains that cases may follow ingestion of shellfish from other parts of the world and recognition of the intoxication will be made easier from the data we report.

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Discussion: Presentations 3, 4, and 5

D. Johnson: I noticed first of all the observation that you have all made about the age of the patients. I have also noticed that in several of the patients there was renal failure to some degree. Have you really looked at all of the records to check on renal function in all of the patients? This might impair excretion of the domoic acid.

J. Teitelbaum: I have had a chance to review 16 of the charts; those are primarily the ones that were hospitalized in intensive care units in the Montreal region, in Prince Edward Island and in Ontario, and there is evidence that almost all of those individuals had reasons to have decreased renal function as you insinuated. Many of them were on drugs including prostaglandin inhibitors; some of them were on beta blockers. They also had concurrent diseases; in addition, the drugs themselves could certainly contribute to decrease the filtration rate. The other thing is that we have 2 cases who had chronic renal failure requiring dialysis, who were under the age of 50, who were hospitalized with severe manifestations in both neurological and cardiovascular systems. There is also an unconfirmed report from the Maritime provinces of another individual with chronic renal failure who had severe memory disturbance and was hospitalized; his presentation was in the psychiatric institution.

M. Ball: Dr. Teitelbaum: In your grouping in the long-term testing of the 12 people into the 2 categories of mild-moderate versus severe, were the ones that were much worse initially the same people who ended up in the second category later on?

J. Teitelbaum: I didn't present that, but there definitely is that correlation. Those who became comatose and who became sickest and took the longest to recover were most severely affected later on.

T. Perl: I'll ask Jeannie Teitelbaum a question. One of my impressions while I was looking through the charts was that seizures was a critical turning point in the presentation of these individuals. Did you have any evidence that if you had seizures you were more likely to have residual neurological deficits?

J. Teitelbaum: Unfortunately, at least 2 of our patients never demonstrated any seizure activity either on EEG or clinically, but are amongst the most severe group, notably patient 6, so I cannot say that there is a definite correlation.

J. Donaldson: In relation to the experimental findings in rats, it seems to me that the data you presented are quite reminiscent of spongioform encephalopathy. I would like to ask if you considered this. Secondly, the effects which you have described are very reminiscent of administration of cardiac glycosides as the cause of hippocampal damage and seizure activity or the encephalopathy.

J. Teitelbaum: I think that Dr. Carpenter is probably better suited to answer the question in terms of whether this resembles spongioform encephalopathy or not.

S. Carpenter: I would say there is no resemblance to spongioform encephalopathy. There is neuronal necrosis but no particular vacuolization of the neurons.

J. Donaldson: The spinal motor neuronal damage?

J. Teitelbaum: We have no histopathological evidence that we have any damage at all in the spinal cord because we received no spinal cord; it has not been analyzed.

S. Carpenter: Actually, I got one little segment of thoracic cord. Thoracic cord has rather few motor neurons and I could not see any abnormality in it. This was from the case that died 3 months after ingestion of the mussels.

Question from the floor: Did anybody look at the muscle for damage, I mean human muscle, histologically?

J. Teitelbaum: No, I am afraid not.

Question from the floor: So the atrophy wasn't documented?

J. Teitelbaum: No, not histologically.

I. Hynie: Trish Perl: Do you know whether any biological material from the patients which was originally submitted for toxicological analysis was preserved so that it can be looked at now when we know that domoic acid is what we are supposed to be looking for?

T. Perl: I think you and Todd have serum specimens from 17 patients. Unfortunately, a lot of these specimens where drawn 1 to 2 weeks into the illness. We have some cerebral spinal fluid from another patient and, in addition, there is some brain that was put in dry ice and sent here. They unfortunately didn't get enough specimens to analyze her brain with the histological sections.

I. Hynie: In hindsight it would appear that what we would really need is urine and stool because it seems, first of all, that most of domoic acid is not absorbed, this is why there is such a discrepancy between the toxicity orally and parenterally. Secondly, it is quite a polar compound, so most of it is either detoxicated and eliminated as glucuronate or sulphate, or is excreted as such, but the concentration in serum probably will be very low. I wonder if anybody ever had any urine?

T. Perl: There was potentially one urine but we were not able to retrace it. It is unfortunate, those specimens are not the kind that are frequently kept by hospital laboratories; next time we will get them.

C. Pinsky: There is probably a rational explanation for the peripheral neuropathies that were seen. Both primary afferents and dorsal neurons are known to have kainic and quisqualate receptors and so do some ventral horn cells. Now the overstimulation that would be occasioned by the actual domoic acid could overstimulate those neurons and the sensory afferents; there are actually receptors on the fibres themselves. I would expect that one could find axonopathy in ventral horn cells as a result and fibre degeneration in sensory afferents but, of course, these peripheral afferents might very likely recover. Do you have comments on whether there was recovery from these neuropathies that was better than the central recovery?

J. Teitelbaum: We have no evidence of the central recovery of the memory function, although those who had some global abnormalities did recover from them. The memory problems did not recover. The peripheral damage did seem to recover in those where we have serial

studies, and there was a recovery of what is likely to be an axonopathy.

G. Glavin: Did you determine levels of domoic acid either in plasma or cerebral spinal fluid, and if so, is there any relationship between these and the severity of the central nervous system and/or the gastrointestinal damage?

T. Perl: Maybe I'll let Dr. Todd answer that. As far as I know, in the 17 specimens from Montreal, the sera and cerebral spinal fluid were negative, but I think he can comment on the assay technique better than I can.

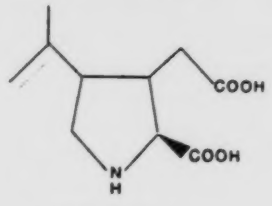
E. Todd: Yes, this is correct, they were negative.

6. *The Toxicology of Domoic Acid Administered Systemically to Rodents and Primates*

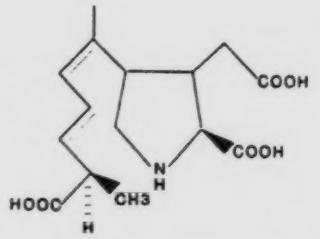
F. Iverson, J. Truelove, L. Tryphonas, and E.A. Nera

In late 1987, approximately 145 individuals, mainly from the province of Quebec, exhibited gastrointestinal distress (nausea, vomiting), and in some cases, neurological symptoms (loss of balance, memory loss, disorientation) after consuming cultivated mussels harvested from river estuaries in Prince Edward Island. Studies conducted by the Health Protection Branch indicated that the mussels contained a substance that produced a scratching syndrome and death in mice administered extracts of toxic mussels prepared for the bioassay normally conducted for paralytic shellfish poison (PSP). Domoic acid (Figure 1) was subsequently identified as a contaminant in the mussels[1,2].

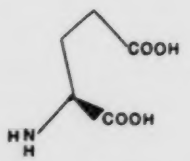
Figure 1
Structure of Kainic, Domoic and Glutamic Acids



KAINIC ACID



DOMOIC ACID



GLUTAMIC ACID

Domoic acid is a known neurotoxic excitatory amino acid (EAA) containing the structure of glutamic acid, the parent excitatory amino acid, and bearing a structural resemblance to kainic acid, another naturally occurring neurotoxic amino acid. The mechanism by which these substances exert their neurotoxicity has been discussed by Olney and others [3]. These compounds have been used as selective brain lesioning agents to produce models for epilepsy, Alzheimer's, and other disorders. Unfortunately, domoic acid is in scarce supply and this has preempted many classical toxicological investigations. For instance, the brain lesions described for domoic acid and the profound inhibition of the brain enzymes choline acetyltransferase and glutamate decarboxylase by domoic acid have been produced after direct, rather than systemic, administration [4]. Nevertheless, the scientific literature can provide data from which assumptions about domoic acid systemic toxicity can be drawn.

Domoic acid is an agonist of the kainic acid subtype of receptor [5]. Kainic acid is more readily available, has been studied more thoroughly, and has several clinical effects on rodents, including excitement, scratching, wet-dog shakes, and convulsions [6,7]. These effects are similar to the atypical signs noted when extracts of toxic mussels were given intraperitoneally (IP) to mice, following the prescribed bioassay procedure for PSP. These clinical signs are, however, substantially different from those expected for PSP, and thus were originally classified as "atypical" or not representative of PSP poisoning.

The pharmacological activity of domoic relative to other EAA receptor agonists varies with the end point, but it is reported to be twice as effective as kainic in depolarizing frog neurons [8]. Other studies indicate that the neuroexcitatory potential of domoic is 3-fold greater than kainic acid, but that its neurotoxic and convulsant activity are equal to that of kainic acid [4]. These studies show domoic acid to be a relatively toxic compound, with a potency equal to, and perhaps greater, than that of kainic acid. This suggests an acute lethal toxicity of about 5 mg/kg (parenterally), the ability to produce hippocampal damage with loss of neurons in area CA3, and damage to the retina [9].

Limited data on a previous human use for domoic acid are also available. In Japan, domoic acid, contained in extracts of the seaweed *Chondria armata*, were apparently used by a group of islanders as a home remedy [10]. Domoic acid was also known to exhibit pesticidal activity since flies died soon after alighting on the seaweed that had been washed ashore. The insecticidal activity of domoic was subsequently studied and it appears to be an effective topical insecticide with activities at least equal to γ-BHC and DDT [11]. Presumably, the insecticidal activity prompted use as an anthelmintic in humans, and studies with seaweed extracts given as an anthelmintic to children reported no toxic effects. Domoic acid, purified from the seaweed, was also given orally to children at 0.5 mg/kg again with no ill effects reported [10]. These data may have suggested to some that domoic acid could not be the agent responsible for the toxic mussel poisoning, despite its known pharmacological and toxicological effects. In fact, kainic acid at the same dose levels was administered to some 28,000 school children with no reported toxic effects. Toxicology studies [12] were, therefore, initiated to compare the dose response of mussel extract with that of domoic acid and to assess the usefulness of the mouse in the bioassay for domoic acid. Approximately 150 mg of purified domoic acid was obtained from the National Research Council and mussel extracts were prepared according to the method for the PSP bioassay. The small amount of purified domoic acid available at the time limited the testing that could be conducted, but subsequent studies at the Health Protection Branch, which will not

be discussed further, indicate that domoic acid is not a mutagen [13], and preliminary studies reveal no teratogenic activity.

The rodent studies were begun by fractionating aliquots of toxic mussel extract and administering the fractions IP to female mice. The scratching behavior was produced only by the fraction containing domoic acid. Isomers of domoic acid are known to exist and these presumably would occur at or near the elution of domoic acid [14, 15]. Several of these domoic acid isomers and analogs apparently incorporate structural changes that reduce toxicity. This does not preclude the possibility that others may exist with enhanced activity. We made no attempt to isolate or test for toxicity of domoic acid isomers, since domoic acid alone could produce the toxicity observed in the mouse and in other experimental animals.

The dose response in mice (Table 1) was determined using toxic mussel extract, and purified domoic acid. The clinical signs displayed by the mice (scratching, rolling, tremors, seizures) were observed using pure domoic acid administered in saline or purified domoic acid added to extract prepared from mussels that did not contain domoic acid (negative extract). The dose response observed in these trials was also similar for each diluent, which is consistent with the assumption that domoic acid is the major toxic species present in the mussels. If the scratching syndrome is selected as the first sign truly representative of the response to toxic mussels, the no-effect level becomes 12 µg (24 ppm in mussels). Deaths occurred at domoic acid levels at and above 100 µg (5 mg/kg). The results indicate that domoic acid acute toxicity is qualitatively and quantitatively equivalent to that of toxic mussel extracts.

Another series of studies (Table 2) probed the toxicity of domoic acid administered orally to mice. The results indicate a variable response with clinical signs (scratching, seizures) displayed over a range of 35 mg/kg to 100 mg/kg body weight. Again the toxic mussel extract and the purified domoic acid in saline elicited a reasonably consistent response with respect to dose. However, it is apparent that the toxicity produced by domoic after oral administration requires about 10-fold more domoic than that utilized in parenteral administration. These results are consistent with unpublished reports from Japan indicating "no effect" in mice given an oral dose of 100 mg/kg.

We also determined the dose response of domoic acid in the rat (Table 3). In this species clinical signs were again consistent between both mussel extract and purified domoic acid, suggesting that domoic acid was the toxic factor in the mussels. Intraperitoneal administration produced seizures and death at 4 mg/kg, while oral administration of mussel extract produced toxicity only after dose levels reached 70 mg/kg, reflecting the situation in the mouse. As expected, the brain lesions observed after IP administration (4 mg/kg), consisted of neuronal degeneration and loss in the CA3 area of the hippocampus.

Since oral administration resulted in large reductions in toxicity, it seemed reasonable to suspect that absorption of domoic acid from the gastrointestinal tract was a significant factor in producing toxicity. We conducted several small excretion studies to determine domoic acid levels in urine and feces from rats and mice given oral doses of domoic acid (Table 4). Results were quite variable, but for both species, the mean results were similar and revealed that almost 100% of the administered dose was recovered in the feces within 36 h. Given orally, domoic acid toxicity in rodents is reduced because it is not appreciably absorbed.

We have recently extended our studies on domoic acid toxicity to the cynomolgus monkey (Table 5). These studies are not yet complete but the preliminary results can contribute to the domoic acid toxicity base. A single IP dose of 4 mg/kg domoic acid is lethal, consistent with the rodent toxicity data. Brain lesions observed were also in the areas observed with rodents. (A complete description of the pathology in rodents and primates will be given later in the Symposium.) Orally, 10 mg/kg domoic acid is tolerated, but unlike the rodent (which cannot vomit), the primate will

Table 1.
Dose Response for Domoic Acid Given IP to Female Mice

Domoic Acid (µg given)	Time to Scratch (min)	Mice Responding	Time to Death (min)	Mice Responding
Toxic Mussel Extract				
12	—	0/3	—	0/3
23	45	1/3	—	0/3
47	38	3/3	—	0/3
117	20	4/4	43	2/4
233	16	6/6	25	6/6
Domoic Acid in Negative Extract				
20	—	0/3	—	0/3
50	29	2/3	—	0/3
100	12	3/3	—	0/3
200	11	3/3	34	3/3
Domoic Acid in Saline				
20	—	0/3	—	0/3
50	32	3/3	—	0/3
100	21	3/3	95	1/3
200	17	3/3	65	3/3

gag and/or vomit shortly after the dose is administered. This occurred at 10 mg/kg and 5 mg/kg but not 0.5 mg/kg. The vomiting, (gagging at lower doses) is probably a direct central nervous system effect of the domoic acid and not a response to gastrointestinal irritation or to other components of the mussel not related to domoic acid. It appears that at oral dose levels where the rodent shows no effect, the primate can absorb enough domoic acid to produce vomiting. Whether this represents a species difference in absorption between rodents and primates must await studies with radiolabelled domoic acid or the application of analytical methods with the capacity to detect domoic acid in blood. The results from the intravenous (IV) dosing studies underline the importance of absorption in producing domoic acid toxicity since only 12.5 µg/kg domoic acid is actually required to produce gagging. Moreover, these results suggest that, once absorbed, elimination or metabolism rather than any blood-brain barriers would seem to be the main factors expected to alter the toxic response to domoic acid. These presumptions will be tested as the primate studies progress.

The information obtained from the literature and from the experiments described above can be used to examine the contribution of domoic acid to the reports of human intoxication following mussel consumption.

- The toxicity of the mussel extracts resides in the chromatographically separated fraction that contains domoic acid.
- Domoic acid alone, in the same concentration found in toxic mussels, can produce the signs of toxicity that are exhibited by animals given toxic mussel extract.
- Domoic acid, and toxic mussel extract containing domoic acid, produce identical lesions in the brains of both rodents and primates.
- The symptoms exhibited by humans consuming mussels are consistent with the pharmacological and toxicological properties of domoic acid.
- The amount of domoic acid consumed by individuals is calculated to be as much as 5 mg/kg or 6 mg/kg based on domoic acid levels in mussels of 960 to 1,280 ppm. These levels will produce vomiting in cynomolgus monkeys given domoic acid and are 10-fold higher than the amounts given as an anthelmintic in Japan.
- Domoic acid is not absorbed well from the gastrointestinal tract. It seems reasonable to assume that any lesion or metabolic condition that enhances absorption may greatly enhance toxicity. Therefore, individuals with gastrointestinal lesions or reduced renal clearance of domoic acid may be at increased risk to domoic acid toxicity. Glutamic acid absorption in humans is characterized by wide individual variations.

In summary, the pharmacological and toxicological properties of domoic acid indicate that it was the agent responsible for the gastrointestinal and neurological symptoms exhibited by individuals consuming toxic mussels in 1987. That all individuals did not appear to be equally affected can be explained by a) the amount eaten, b) levels of domoic acid in the mussels, and c) varying susceptibility due probably to any condition that would have increased domoic absorption and/or prevented elimination. Neurological symptoms were most often encountered in the elderly and the most severe cases were reported in this group. A combination of the factors listed above may have predisposed these individuals to the toxic effects of domoic acid.

Table 2.
Dose Response for Domoic Acid (Oral Administration to Mice)

Domoic Acid (mg/kg)	Response	Time to Death (in min)
Toxic Mussel Extract		
35	scratch	-
35	none	-
47	scratch	26
58	none	-
80	none	-
100	none	-
104	seizure	28
Domoic Acid in Saline		
20	none	-
28	none	-
35	scratch	-
65	scratch	-
68	seizure	49
70	none	-
80	none	-

- = no death

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Table 3.
Response of Rats to Oral and IP Administration of Mussel Extract And Domoic Acid

Domoic Acid (mg/kg)	Response	Time to Death (in min)
Toxic Mussel Extract (IP)		
2	none	-
4	seizures	71
4	seizures	72
4	seizures	-
7	seizures	29
8	seizures	21
Domoic Acid In Saline (IP)		
2	none	-
2	scratch	-
4	seizures	112
8	seizures	23
Toxic Mussel Extract (oral)		
60	none	-
70	mastication	-
80	none	-
80	seizures	32
82	seizures	30
82	seizures	44

- = no death

Table 4
Fecal Excretion of Domoic Acid (in 48 hours)

Species	Number	Amount (%)
Rat	4	98 ± 12
Mouse	4	102 ± 17

Discussion

A . Tasker, Charlottetown: Dr. Iverson, first of all, a quick comment on the systemic toxicity. We have also done dose-response relationships looking at domoic acid in various forms and I would just like to state that, although we measure our toxicity differently, we have a 7-point behavioral scale that we evaluate it on. The ED-50s look to be almost identical with your own, that is just confirmation.

F. Iverson: Very good, thanks.

A. Tasker, Charlottetown: The other question I have is in the oral studies. Were these animals fasted prior to administration or were they not?

F. Iverson: Studies were conducted with both fasted and non-fasted animals.

A. Tasker, Charlottetown: And regardless whether they were fasted or not, you didn't see any difference in the bioavailability?

F. Iverson: No, we saw a great range in dose response and I think that is the thing I wanted to bring out; there appeared to be quite a range in absorption between these animals. The excretion studies we did indicate that

there is quite a variation. I think you can appreciate that there is quite a difference between going from, say, an oral dose of domoic acid in saline to PSP bioassay material, which is really just homogenization of a batch of mussels, heating, and spinning to get rid of the bigger particles and then administration of this material. There are some differences in there but, I think, it shows clearly that, as you found out, domoic acid seems to account for most of the toxicity. But there is a range in absorption, quite a variability. That is why we got effects at 35 mg/kg in one case and 177 mg/kg in another.

A. Tasker, Chartlottetown: Yes, I would certainly confirm that. We see basically the same sort of thing with oral administration in rats in that there seems to be a great deal of variability in terms of the bioavailability which doesn't seem particularly well correlated with any other factor, at least, as far as we can see.

F. Iverson: Good, I am glad to hear that.

A. Novelli: I have a comment about your studies with the injection of domoic acid in toxic mussel extract. It seems that domoic acid in saline is somehow more important than the domoic acid in the mussel extract. However, you also say that the mussel extract has been concentrated in order to achieve higher concentration. Now I wonder about the concentration of bivalent ions in the mussel extract, even a normal one, because a very high concentration of bivalent ions has been reported to inhibit the action of excitatory amino acids in hippocampal neurons and other systems. So maybe you got a lower stimulation of your effect because of some interference by a high level if ions. I am talking about magnesium, cadmium, zinc, whatever can be present.

F. Iverson: Well, that is a possibility. We didn't have to concentrate for the IP studies and I think we have just discussed the oral studies. We always went back and measured domoic acid again to make sure that it was still around in the concentration that we began with and it was measured on the material that we were actually giving. But yes, there probably are higher concentrations of ions in the concentrate and if there was any effect then the toxic muscles would , in fact, be less toxic than domoic acid, which is the safe way anyway. But yes, you are right, there possibly is a slight difference there.

G. Debonnel, McGill: For your monkeys following the intravenous administration of domoic acid, I got the impression that you had a kind of correlation between the doses and the time of onset of the symptoms: the higher the dosage, the longer the period between the injection and the beginning of the symptoms. Do you have any comments on that or is it just an impression?

F. Iverson: I know there is nice dose response. We could go back and look at the slide. I don't know if there is a difference in time between the onset of response or if there is any correlation there. I know there was certainly a very nice dose-related correlation in the duration of the response. At the lowest doses it would be a couple of minutes or maybe less on a gag-type response and up to over 119 minutes for high doses. So there was a very nice graded dose response in the duration of the effects that took place.

G. Debonnel, McGill: If I recall from the lower dose, the time of onset was about 4 minutes and for the higher dose about 7 minutes.

F. Iverson: Well, I would say that there is certainly some variation in the onset time, I think 8, 4, 12, 8, and 17 minutes. It seems to be very quick and that seems to be a good average. With duration times though, the dose response goes up very nicely: 1, 4, 38, 61, 119. I think that is probably what counts. The onset times are very short, but this is not surprising since the material was given intravenously.

Table 5.
Dose Response for Domoic Acid in Cynomolgus Monkeys

Domoic (mg/kg)	Pathology	Route	Response
0.5		oral	none
5.0	-	oral	vomit (onset 12 h, duration 80 min) mastication, yawning, fine tremor
10.0	-	oral	vomit (onset 1 h, duration 450 min) mastication, fine tremor
0.00625		IV	none
0.0125		IV	gag (onset 8 min, duration 1 min)
0.025		IV	gag (onset 4 min, duration 4 min)
0.05		IV	gag (onset 12 min, duration 38 min)
0.20		IV	gag (onset 8 min, duration 61 min)
0.50	-	IV	gag (onset 17 min, duration 119 min)
4.0	+	IP	vomit, mastication, rigidity tremors, incoordination, death

7. *Chemistry, Biology, and Toxicology of Domoic Acid and its Isomers*

J.L.C. Wright, C.J. Bird, A.S.W. de Freitas, D. Hampson, J. McDonald, and M.A. Quilliam

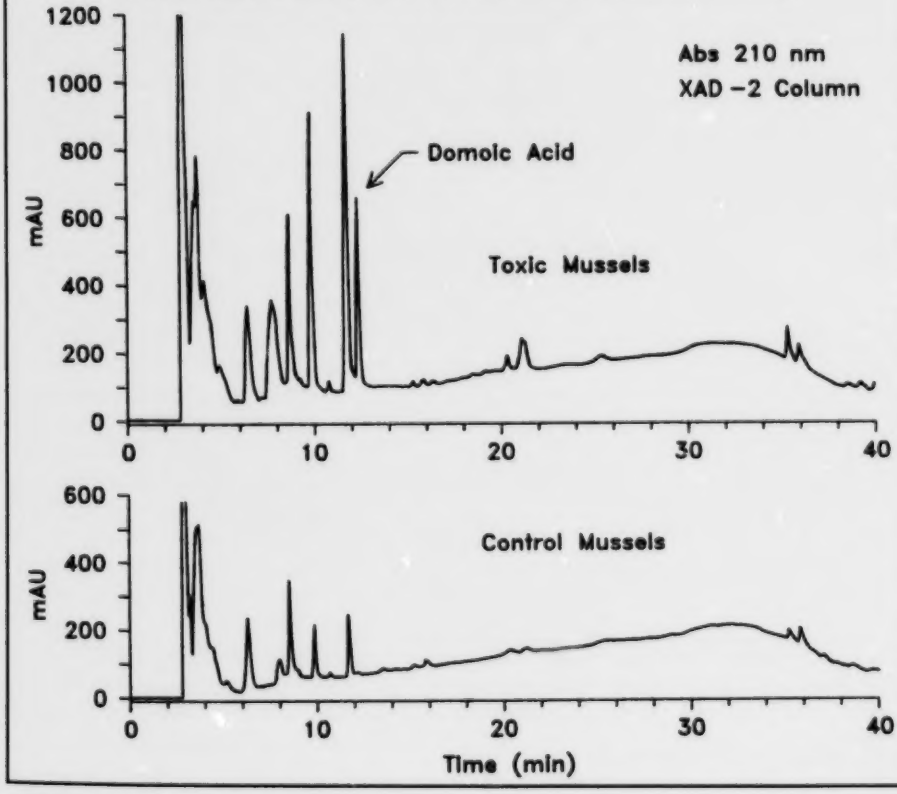
Abstract

The causative agent of toxicity in cultured mussels from a localized area of eastern Prince Edward Island has been identified as domoic acid, a neuroexcitatory amino acid. The toxin was isolated by a number of different bioassay-directed separation techniques including high-performance liquid chromatography (HPLC), high-voltage paper electrophoresis (HVPE), and ion-exchange chromatography, and characterized by a number of spectroscopic techniques including ultraviolet, infrared, mass spectrometry, and nuclear magnetic resonance. The isolation and purification methods are described in detail and some new analytical data for domoic acid are reported. A plankton bloom at the time of the outbreak gave positive mouse bioassays and consisted almost entirely of the pennate diatom, *Nitzschia pungens* f. *multiseries*. A positive correlation was found between the number of *N. pungens* cells and the concentration of domoic acid in the plankton. *N. pungens* f. *multiseries* isolated from Cardigan Bay produced domoic acid *de novo* during stationary phase culture at levels (1 to 10 pg/cell) comparable to values estimated for *N. pungens* in the plankton samples. We conclude that *N. pungens* was the major source of the domoic acid in toxic mussels in eastern Prince Edward Island. The recurrence, in November 1988, of a monospecific bloom of *N. pungens* and the presence of domoic acid in plankton and mussels reinforces this conclusion.

Introduction

In late 1987 a mysterious and serious outbreak of food poisoning occurred in Canada. Symptoms of the poisoning included vomiting and diarrhea, followed in some cases by confusion, memory loss, disorientation, and even coma. Two elderly patients died and in some other severely affected cases the neurological symptoms still persist. Epidemiologists from Health and Welfare Canada soon attributed the illnesses to restaurant meals of cultured blue mussels (*Mytilus edulis* L.). Using the Association of Official Analytical Chemists' mouse bioassay for "red-tide" paralytic shellfish poison (PSP) [1], Health and Welfare Canada and Fisheries and Oceans scientists demonstrated that these mussels did contain toxic material and, furthermore, were able to trace the problem to mussels harvested from a localized area of eastern Prince Edward Island. Intraperitoneal injections of acidic aqueous extracts of suspect mussels into mice [1] caused death with some highly characteristic and unusual neurotoxic symptoms, very different from those of PSP and other known toxins. At that point it was not known whether the toxic agent was a man-made pollutant or a natural toxin. An intensive investigation by a team of scientists at the National Research Council laboratory in Halifax established the toxic agent as domoic acid [2]. In the following months, a large-scale collaborative effort involving scientists from Fisheries and Oceans, the National Research Council, and the University of Prince Edward Island established that the toxin had been produced by the marine pennate diatom *Nitzschia pungens* f. *multiseries* [3]. Further chemical investigations of mussel and phytoplankton extracts have uncovered a series of domoic acid isomers and the relative biological activity of the isomers will be discussed.

Figure 1
Typical Dose-Response Curve Relating Time-of-Death to Weight-Equivalent Whole Mussel Tissue Used to Provide Extract for Mouse Bioassay



Results and Discussion

Toxic mussels and control (non-toxic) mussels were supplied by Fisheries and Oceans (Charlottetown, Prince Edward Island). In all cases extracts of both toxic and control mussels were taken through identical procedures. This was done to distinguish the effects of the unknown toxic factor in the bioassay from inherent salt toxicity in some fractions, and from possible interference due to other factors such as chromatographic mobile phases. Furthermore, this parallel approach would highlight additional or unusual signals present in chromatograms and spectra of only toxic mussel fractions. Strict control over the dosage used in the mouse bioassay was an important element of the procedure. A preliminary dose-response curve (see Figure 1) indicated a narrow range, less than 1 order of magnitude, for the bioassay of the toxin(s). Extracts representing 0.3 to 2.5 g-equivalent of original wet tissue resulted in times of death (TOD) of 100 to 10 min after intraperitoneal injection, respectively. Volumetric control was used to afford an intraperitoneal dose in the range of 1.2 to 2.5 g-equivalent of original tissue, independent of the actual amount of non-toxic material extracted. In addition, it was important to verify that the symptoms induced in the bioassay by toxic fractions were exactly the same as those observed with extracts of toxic mussels.

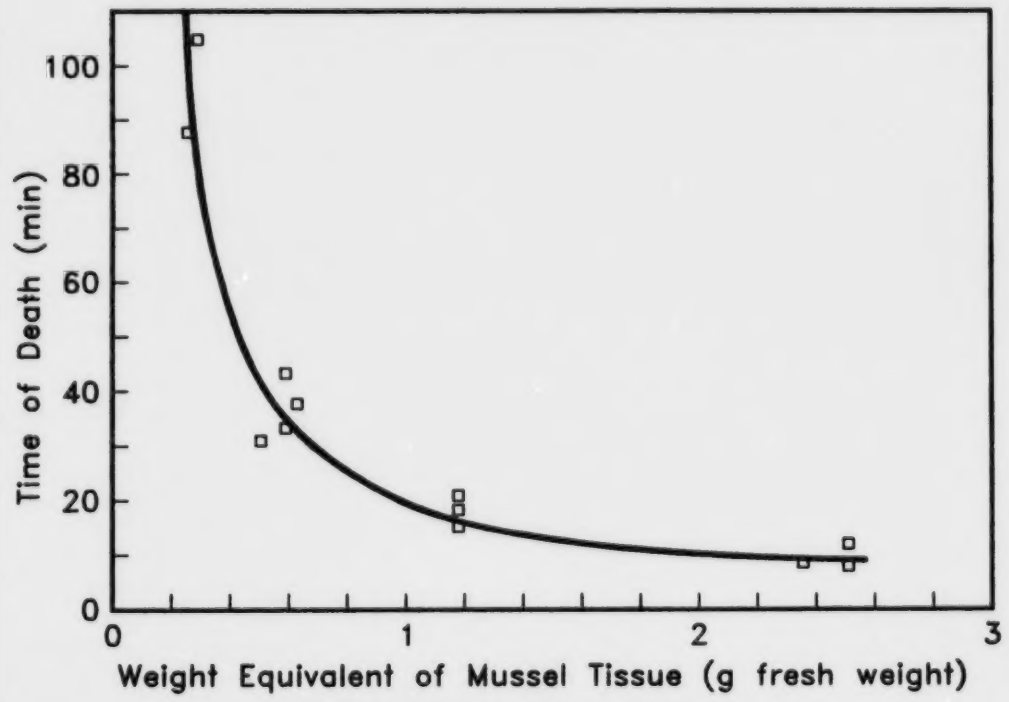
In initial experiments the digestive glands were separated from the remaining mussel tissue. Each tissue type was extracted with methanol:water (7:3 v/v), concentrated, and then partitioned against dichloromethane. In no case was organic-soluble toxicity found. Aqueous extracts of both tissue types from control mussels displayed no toxicity but the digestive-gland extracts of toxic mussels were found to be toxic. The remaining tissue of toxic mussels displayed some low-level toxicity but this was attributed to incomplete separation of the two tissue types. However, in the interest of timesaving during very large-scale extractions, the entire mussel was processed.

In the initial search for the toxin, several methods of separation (HPLC, HVPE, and ion-exchange chromatography) were selected to avoid the possibility that in any 1 process the toxin might be obscured by other non-toxic compounds present in the extract. Furthermore, toxic fractions isolated by one scheme were cross-analyzed by another to verify that they were the same material, thus providing an internal check on the validity of the separation schemes.

Based on HVPE results, the premise was established that the toxic material was possibly an acidic peptide. A reversed-phase HPLC method was employed using a C_{18} stationary phase and gradient elution with water-acetonitrile mixtures containing 0.1% trifluoracetic acid (TFA) to suppress ionization of the carboxyl groups. Detection was provided by a diode-array detector (DAD), which allowed continuous acquisition of UV-visible spectra. The HPLC profile of the toxic HVPE fraction obtained from the XAD-2 column, compared with the equivalent fraction from control mussels is shown in Figure 2. Initial runs were monitored by UV absorption at 210 nm since most peptides and organic acids absorb at this wavelength. The chromatogram for the toxic fraction showed a unique peak at retention time 12.1 min (peak D). The equivalent region in the control chromatogram showed no peak.

The UV-visible spectrum acquired at the apex of this peak revealed a single absorption maximum at 242 nm, suggesting the molecule contained 2 conjugated double bonds. It was possible to collect fractions corresponding to peak D, as well as other regions of the chromatogram, for bioassay and chemical analysis by preparative HPLC. However, all the fractions obtained in the first series of preparative runs displayed toxic effects both in the control and toxic samples, although the symptoms were

Figure 2
HPLC Traces for Extracts of Toxic* and Control Mussels



* toxic extract contains a peak (RT 12.5 min) not present in the control extract

different from those induced by toxic mussel extracts. This problem of general toxicity was resolved by replacing the rodenticide trifluoroacetic acid used in the mobile phase with 1% acetic acid. The peak D fraction was then found to be toxic to mice, with the correct symptoms. All the other fractions from aqueous methanol extracts were non-toxic, even at a 5-fold higher dose in terms of weight equivalents of original tissue. When the toxic HPLC fraction was subjected to HVPE, a yellow band was observed at a position corresponding to where toxicity was previously observed. Bioassays of aqueous extracts of slices of this electrophoretogram revealed that only the yellow band was toxic. Conversely, this band, obtained by preparative HVPE of crude extracts, gave an HPLC peak at the same retention time as peak D, and the same UV spectrum. The fact that such different fractionation schemes yielded the same toxic factor was very important to the certainty of the result.

Analysis of mussel extracts with a conventional amino acid analyser revealed an unusual peak present only in toxic samples. The compound eluted between methionine and isoleucine; the ninhydrin-reaction product absorbed strongly at 445 nm, consistent with a proline derivative.

The IR spectra of the early toxic fractions from the preparative HPLC runs were highly variable, due to dependence on the pH at which the films are deposited. Also, the spectrum of the toxin was partly masked by absorption bands due to acetate or trifluoracetate salts, contaminants originating from either acetic or trifluoroacetic acid used in the chromatographic separations. Both of these complications were removed by depositing the films from a solution of 0.1M HCl. Acidification ensured a uniform and essentially complete protonation of the toxin molecules (shown by the absence of further spectral change upon evaporation from additional quantities of HCl). When purer samples of the toxin were obtained, the IR spectra showed absorption bands characteristic of non-ionized COOH (1,215 and 1,715 cm^{-1} and a broad band with maxima at 3,000, 2,750, and 2,600 cm^{-1}), ionized COO: (1,400 and 1,612 cm^{-1}), NH_2+ (1,565-1,585 and 1,950 cm^{-1}), *trans* — CH=CH - (966 cm^{-1}), olefinic CH groups (3,030 cm^{-1}), and aliphatic CH groups (2,980, 2,935, and 2,880 cm^{-1}).

Fast-atom bombardment mass spectrometry (FAB/MS) indicated the significance of an ion at m/z 312 in the positive ion spectra. This signal was absent from spectra of control samples which, together with an ion at m/z 310 in the negative ion spectra, suggested a molecular weight of 311 Da for the toxin. An even-electron ion at m/z 312 must contain an odd number of nitrogens; in addition, tandem mass spectrometry indicated the presence of at least 2 carboxyl groups. Accurate mass measurement of the signal at m/z 312 gave a value of 312.146± 0.003 Da. Eleven molecular formulae are consistent with this value. However, by applying the additional restriction of an odd number of N atoms plus at least 4 oxygen atoms (at least 2 carboxyl groups) the possibilities are reduced to just 2 $C_{15}H_{22}NO_6$ for the protonated molecule. Further MS experiments were more consistent with a tricarboxylic amino acid of molecular formula $C_{15}H_{21}NO_6$.

Early ^1H NMR experiments using THF-d_8 had yielded broad spectra, although, in retrospect, the domoic acid resonances are identifiable. The spectra in DMSO-d_6 showed the compound to be largely free of other organic impurities and enabled resonances characteristic of a vinylic methyl group (1.81 ppm, doublet) to be identified. The connectivity of the olefinic protons, H-5', and the allylic methyl group was determined from their coupling patterns and a COSY experiment, establishing the partial structure CH_3-CH-CH=CH-CH=C-CH_3.

Throughout the structure analysis, the UV, IR, MS, and NMR data were continually updated and used to search the Chemical Abstracts and its accompanying Registry File on the STN International on-line database. The search, in which the partial structual data, toxicological data, and the marine origin of the toxin were used together, led at first to a long list of compounds. However, as the structural information became progressively more detailed, this list shrank rapidly until the neuroexcitatory amino acid domoic acid and its isomers emerged as likely candidates.

Meanwhile, ^1H NMR spectra of the toxin were found to sharpen when D_2O was added to the DMSO-d_6 solution, and indeed in D_2O at pH 3.1 a ^1H spectrum was obtained that agreed in every respect (chemical shifts to 0.02 ppm, spin-spin couplings to 0.2 Hz) with detailed published data for domoic acid [4, 5], taking into account that the published data were obtained at an unspecified pH. It also became apparent that the IR and MS data for the toxin were consistent with the domoic acid structure, and further spectral data were collected that completed the final identification.

Although chromatographic separations of aqueous methanol extracts showed no other detectable toxins, it was still important to establish that domoic acid accounted for all of the toxicity in the original mussel tissue. In the days following, many hours of painstaking work established this toxicity balance.

One further point must be mentioned. Domoic acid was first identified in crude extracts of certain seaweeds found in Japan [6], and was used there as a folk-medicine remedy for intestinal worm infestation. This fact apparently argues against this compound as a toxin. However, when the very high levels concentrated in the toxic mussels (up to 900 µg/g wet weight) are taken into account, with the implication that as much as 0.2 to 0.3 g must have been ingested if these mussels were consumed as a main dinner course, the difference between therapeutic and toxic doses is easily accounted for.

The neurotoxicity of domoic acid arises from its effect as a potent glutamate agonist [7]. Domoic acid can be considered to be a conformationally restricted form of glutamic acid that disrupts normal neurochemical transmission in the brain by binding to certain glutamate receptors of normal cells. This results in continuous stimulation in neurons, leading to rupture and the eventual formation of lesions.

Since domoic acid was not detected in mussels from elsewhere in Prince Edward Island, it is unlikely that it is a metabolic product of the mussel itself. Moreover, toxic shellfish held in tanks of clean water for several weeks steadily purged themselves of domoic acid, a phenomenon often associated with dietary products of shellfish [8]. As efficient filter-feeding bivalves, mussels

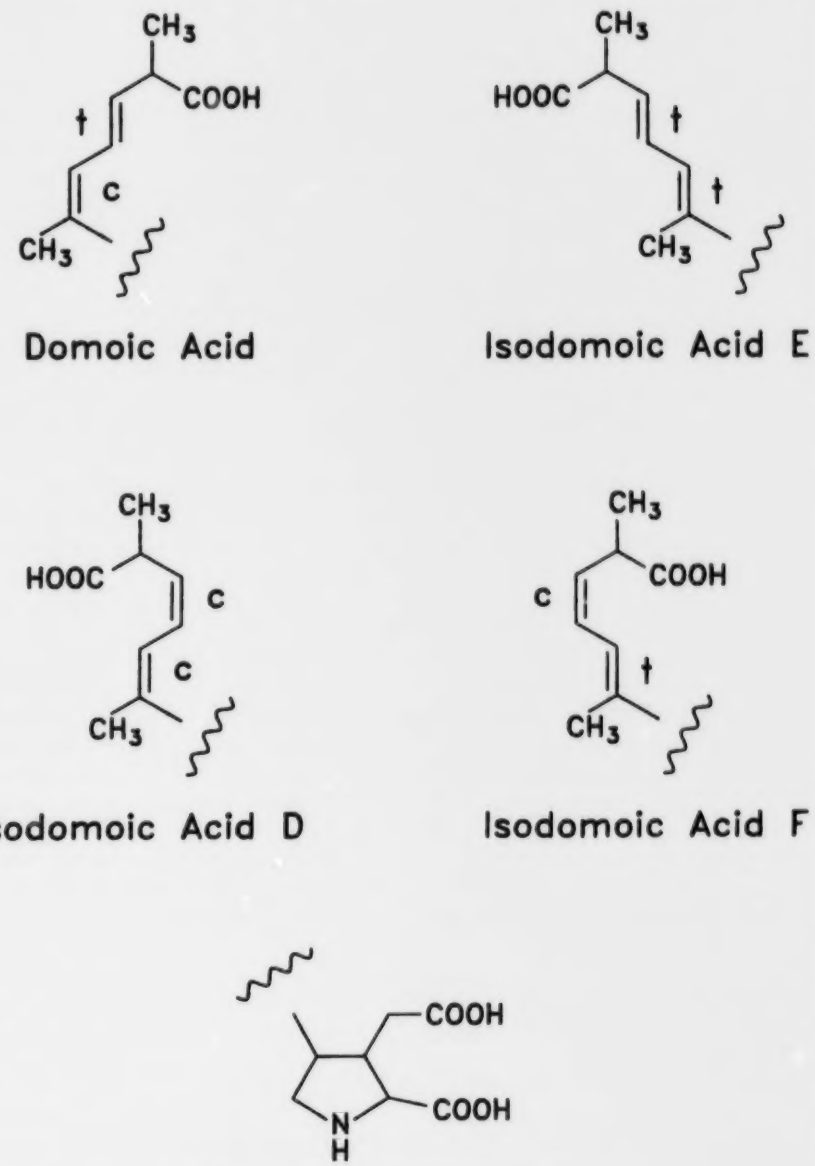
process huge volumes of water relative to their body size [9], and at least some of the toxin in their tissues could be derived from low (undetectable) levels of domoic acid in sea water. However, the food vector is probably much more important than the water vector to account for the substantial quantities of domoic acid found in contaminated mussels. It is estimated that the total amount of domoic acid in mussels from the affected area was probably in excess of 6 kg, with essentially all of the toxin body burden associated with digestive gland tissue.

The major food items for cultured mussels are phytoplankton and other particulate detrital organic material, such as fragmented seaweed tissue from benthic macrophytes and particulate organic material of terrestrial origin in the marine environment by surface-water run-off. Plankton tows made in early December 1987 by L.A. Hanic (University of Prince Edward Island) revealed an intense diatom bloom in the affected area. This diatom was subsequently identified as *Nitzschia pungens* Grun. f. *multiseries* Hasle. When injected in mice, extracts of the plankton tow samples produced symptoms identical to those produced by extracts from toxic mussels. As well, all toxic mussels harvested in December 1987 showed engorged digestive glands containing identifiable shell remnants of *N. pungens*; this was also true of toxic mussels harvested in late October and November, although no information is available on the plankton composition at the time. We observed domoic acid in plankton from the affected areas at levels sometimes in excess of 1% dry weight [10], sufficient to account for the levels observed in toxic mussels. This strong evidence that *N. pungens* was the major source of domoic acid in the toxic mussels is further supported by the demonstration of *de novo* production of domoic acid by laboratory cultures of *N. pungens* isolated from the affected area [10, 11].

Little is known about the autecology of *N. pungens*. It is distributed almost worldwide in coastal waters[3], but statements on its temperature and salinity preferences are based largely on distributional records. We are not aware of any previous reports of this diatom forming dense, persistent unialgal blooms. If *N. pungens* was present at bloom levels in eastern Prince Edward Island as early as October 1987, as seems to have been the case, it is difficult to explain why this bloom persisted and intensified over the 3-month period from October to December. A possible factor is the insecticidal property of domoic acid [12], which might suggest that it could also act as an antifeedant, thus inhibiting grazing by planktonic crustacea.

We conclude that the pennate diatom, *N. pungens* f. *multiseries*, was the major source of the domoic acid contained in the digestive glands of toxic mussels from the Cardigan Bay region of Prince Edward Island in late 1987 and 1988. In view of the global distribution of *N. pungens*, and the strong possibility that there may be other microalgal sources of domoic acid, contamination of

Figure 3
Domoic Acid and Three Geometrical Isomers* Isolated from Mussel Tissue



* these isomers can also be prepared by photolysis of domoic acid

samples from the affected area must have been associated with the *N. pungens* of the plankton. The recurrence of domoic acid containing *N. pungens* in Cardigan Bay in late autumn 1988, and the demonstration of *de novo* production of domoic acid by cultures of *N. pungens* isolated from the affected area, confirm this conclusion.

Detailed HPLC-DAD analyses of contaminated mussel tissue revealed the presence of at least 3 other peaks close to domoic acid and displaying the same UV-absorption spectra. Large-scale extraction and repeated HPLC chromatography yielded enough of these new compounds for structure elucidation and biological testing. All the new compounds were shown to be geometrical isomers (Figure 3) of domoic acid [13]. These isomers are easily prepared from domoic acid by irradiation with UV light [13] and since they occur in phytoplankton extracts as well, they probably arise from irradiation of domoic acid with sunlight.

The differences in neurotoxicity between kainic and domoic acid [14], together with the recent report [15] that acromelic acid A is even more potent than domoic acid, strongly suggest that the nature of the side chain is important to the biological activity of the compound. Consequently, it was of interest to compare the biological activity of these photoisomers with domoic acid. We examined the relative binding strength of the *cis,cis* isomer with rat forebrain membrane and obtained an IC_{50} value of 100 nmol compared with IC_{50} value of 5 nmol for domoic acid itself. These data indicate that differences in the geometry of the side chain can considerably reduce the efficacy of generalized binding to rat forebrain membrane. In a series of voltage-clamp experiments with cultured mouse hippocampal neurons, the relative inward currents generated following brief exposure to domoic acid or its isomers were measured. The currents are associated with an increase in membrane conductance to Na^+ and K^+ ions and would evoke a strong excitation of neurons.

These studies have shown that the nature of the side chain has a profound effect on the activity of the compound. The voltage-clamp studies show the trend: domoic acid > dihydrodomoic acid > isodomoic acid D (*cis:cis*) ~ isodomoic acid F (*trans:cis*) > isodomoic acid E (*trans:trans*). A similar but not identical trend is observed with the binding studies. The results of these structure activity studies clearly highlight the specificity of the kainate receptor for certain substrates. Work is in progress at other laboratories to isolate and characterize the receptor protein and our current studies will be invaluable in delineating the active site of the kainate receptor and may eventually lead to the development of a reversible inhibitor or antagonist that would counteract the neurotoxic effects of domoic acid.

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8. *Determination of Domoic Acid in Seafoods and in Biological Tissues and Fluids*

J.F. Lawrence

Abstract

Domoic acid is extracted from mussel tissue using the Association of Official Analytical Chemists procedure for paralytic shellfish toxins. This involves boiling the sample for 5 min with O.1*N* HCl then cooling and centrifuging. An aliquot of the supernatant is diluted 10 to 100 times with water, filtered and analyzed by reversed-phase liquid chromatography with a mobile phase of acetonitrile-water (12:88) at pH 2.5 and absorption detection at 242 nm. The detection limit is about 1 mg/kg domoic acid in seafood samples. The method was successfully used in collaborative studies and a survey of 44 different commercially purchased shellfish products from areas outside of Prince Edward Island showed no domoic acid greater than 1 mg/kg. The same method was applied to urine and feces from monkeys and blood (serum) from humans. The method was unsuccessful for urine and blood which required additional cleanup before analysis. The method worked well for feces at domoic acid levels greater than 1 mg/kg.

Introduction

In early 1988 the Food Research Division, in collaboration with the Atlantic Research Laboratory of the National Research Council in Halifax, developed a routine method for domoic acid in shellfish tissue. The method is based on the mouse bioassay procedure for paralytic shellfish poisons (PSP), collaboratively studied by the Association of Official Analytical Chemists [1], which was found applicable to domoic acid at levels greater than *ca* 40 mg/kg in wet mussel tissue. We applied this method to the quantitative determination of domoic acid by high-performance liquid chromatography (HPLC) and found that levels as low as 1 mg/kg could be detected. The HPLC method is described herein with application to shellfish and biological tissues and fluids such as urine, feces, and blood.

Experimental

Domoic acid was isolated from contaminated mussel tissue, purified and characterized as described elsewhere[2,3]. This was used as the analytical standard.

Figure 1
Typical Chromatograms of Domoic Acid and Contaminated Mussel Samples

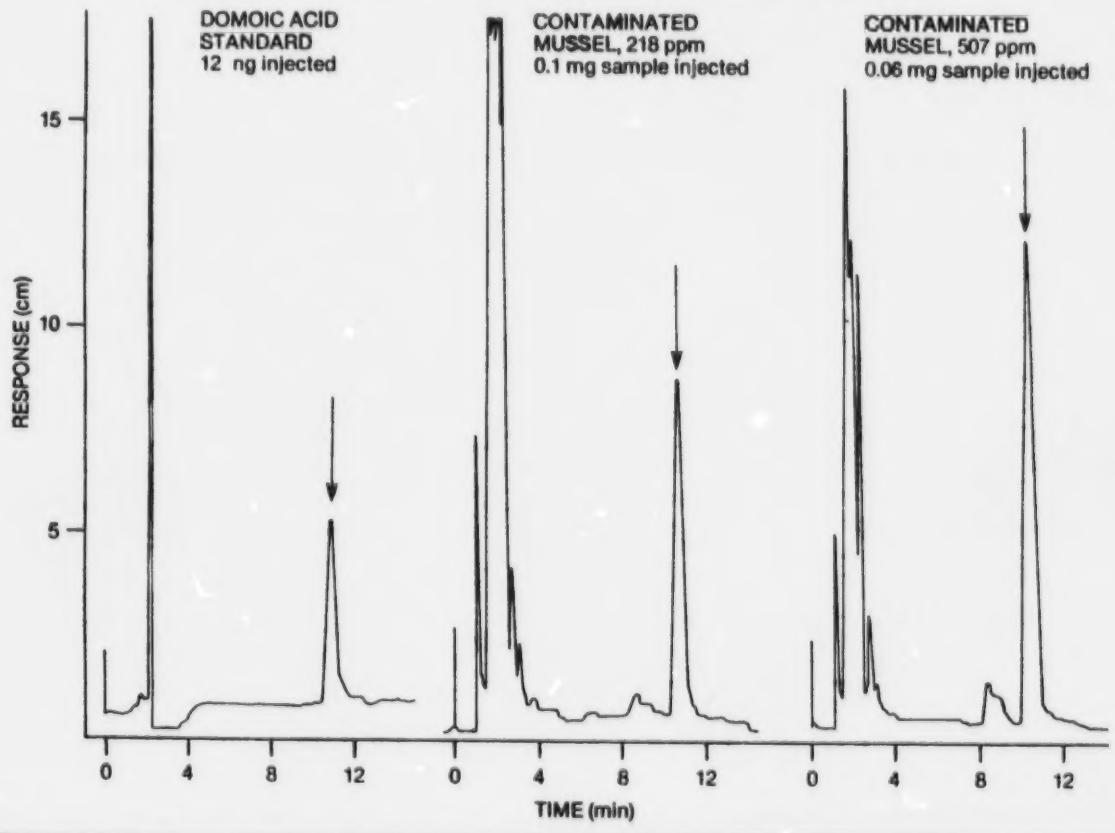
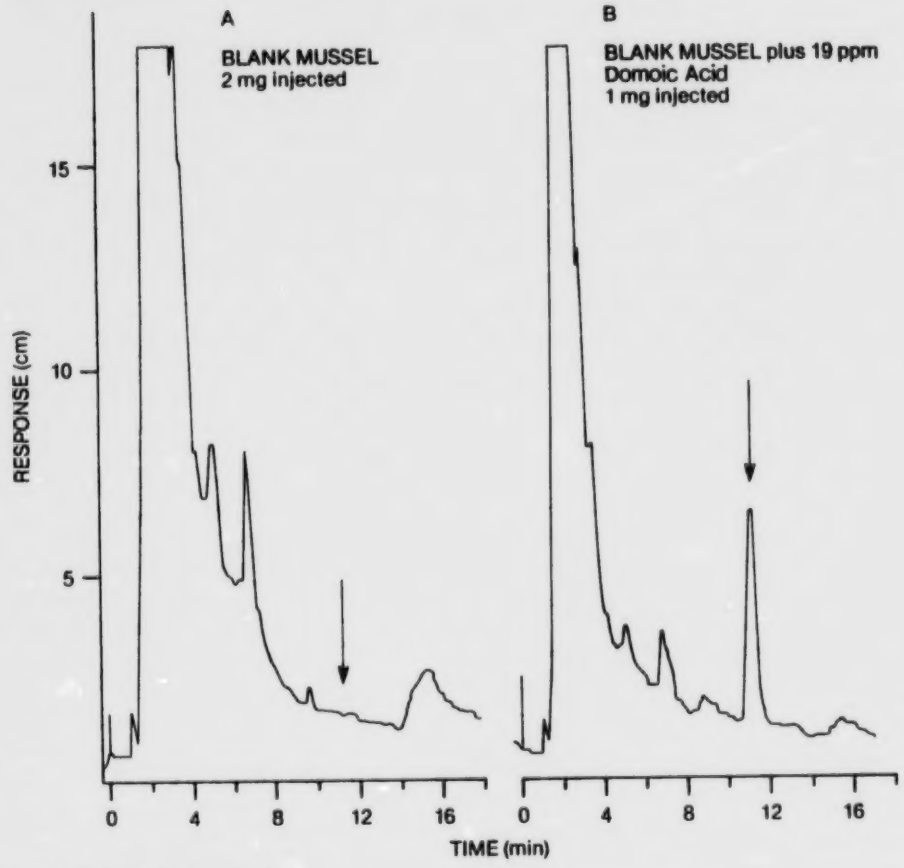
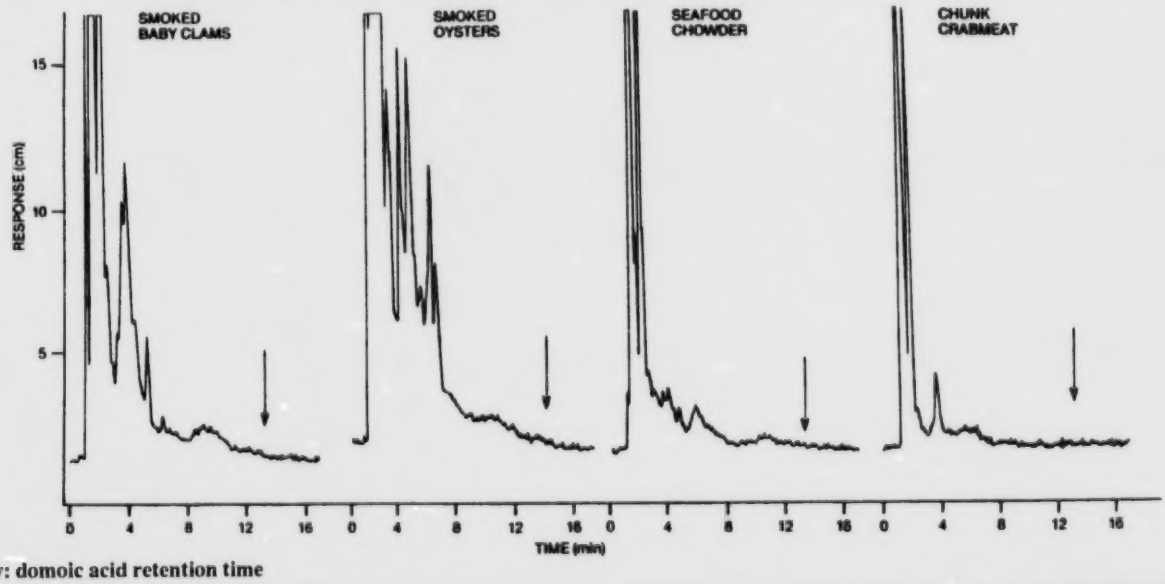


Figure 2
Chromatograms of a Blank Mussel Sample and a Blank Plus 19 mg/kg Domoic Acid



Arrow: domoic acid retention time

Figure 3
Chromatograms of Different Seafood Products (0.5 mg of Equivalent Sample Injected)



Arrow: domoic acid retention time

Table 1.
Shellfish Samples Analyzed for Domoic Acid

Mussels	- smoked	**Chowders**	- clam
	- in sauce		- seafood
	- in brine		
	- marinated	**Other**	- crab
	- in vinegar		- shrimp
			- lobster paté
Clams	- baby		
	- smoked		
	- chopped		
Oysters	- whole		
	- smoked		

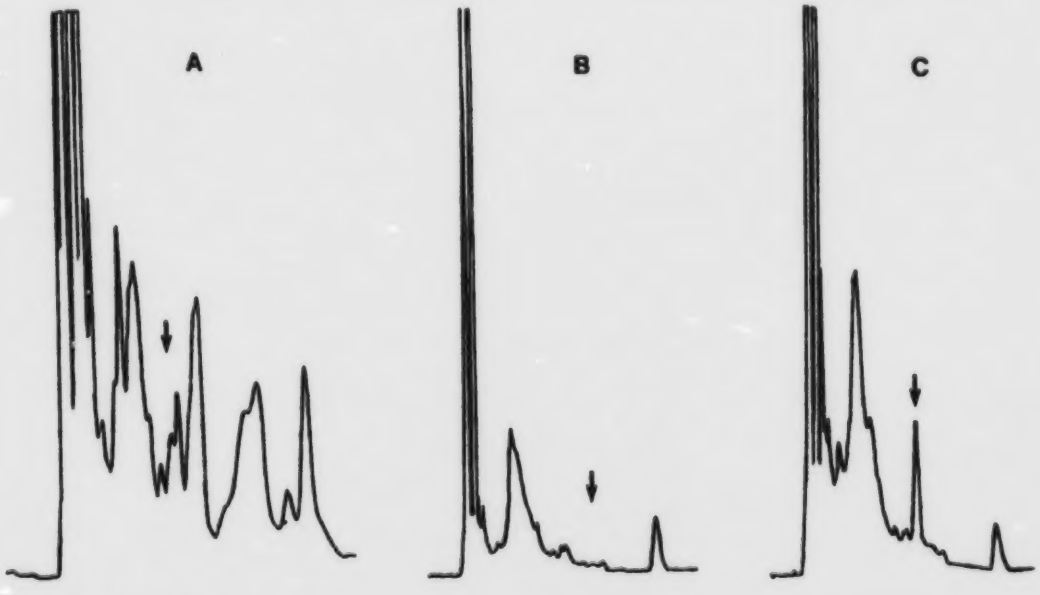
Table 2.
Results of Collaborative Study on Domoic Acid

Number of laboratories: 10
Number of samples: 7
Concentration of domoic acid in the samples:

A) 0
B) 14 ppm
C) 18 ppm
D) 100 ppm
E) 250 ppm

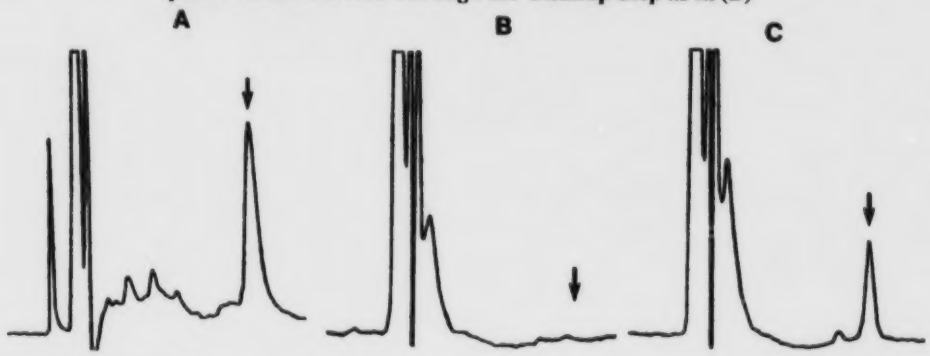
Percent recovery - (average) 72%
Number of outlier values - 2 out of 70
Within laboratory repeatability (CV) - average, 3.4%
Between laboratory repeatability (CV) - average, 13.4%

Figure 4
Chromatograms of Monkey Urine Sample Before (A) and After (B) Additional Cleanup Chromatogram (C) is a Spiked Urine Carried Through the Cleanup Step as in (B)



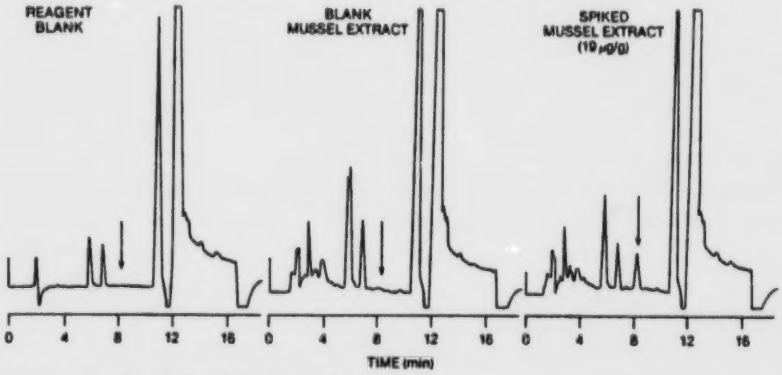
Arrow: retention time of domoic acid

Figure 5
Chromatograms of Human Serum Sample Before (A) and After (B) Additional Cleanup; Chromatogram (C) is a Spiked Serum Carried Through the Cleanup Step as in (B)



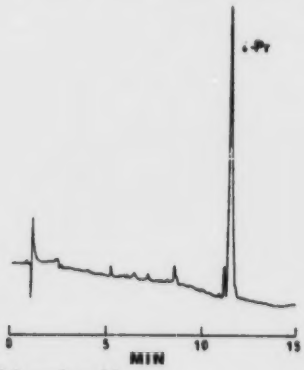
Arrow: retention time of domoic acid

Figure 6
Chromatograms of Reagent Blank, Control Mussel Extract and Spiked Mussel Extract After Cleanup and PITC Derivatization



Arrow: retention time of domoic acid

Figure 7
Chromatograms of Contaminated Mussel (308 mg/kg) Extract After Cleanup and Esterification



i-Pr = isopropyl triester of domoic acid

The method of extraction was exactly as described elsewhere[4]. Briefly, 50 g of shellfish tissue was mixed with an equal volume of 0.1*N* HCl and boiled gently for 5 min. The mixture was cooled quickly in a refrigerator and the contents adjusted to 100 mL volume. The mixture was centrifuged and an aliquot of the supernatant diluted 10 to 100 times then filtered for HPLC analysis.

HPLC was carried out on a Supelcosil C18 column with a mobile phase of acetonitrile-water (12:88) at pH 2.5 (adjusted with 2% H_3PO_4) at 1 mL/min flow rate. Detection was by UV absorption at 242 nm.

Results and Discussion

Figure 1 shows typical chromatographic results obtained with contaminated Prince Edward Island mussels while Figure 2 shows a spiked blank sample at 19 mg/kg. Under these conditions, less than 2 mg/kg domoic acid could be detected in shellfish. Figure 3 shows typical results for seafood products purchased locally. Table 1 lists the types of products analyzed. Forty-four different samples were included consisting of both imported and domestic products. The method was found to work well for all seafood samples analyzed.

In order to ensure that results from the many different laboratories involved in domoic acid analysis were comparable, an interlaboratory collaborative study was organized and carried out. Table 2 lists the results of the study. Only 2 outlying values were observed and the method had acceptable recovery near the regulatory guideline level of 20 mg/kg and good between-laboratory repeatability over all concentrations studied. This indicated that the 10 participating laboratories are capable of generating equivalent domoic acid results.

The shellfish procedure was applied to feces and urine from monkeys. The method worked well for feces but required additional sample cleanup for urine. The cleanup involved ion exchange chromatography of the acid extract on a strong anion exchange column followed by reversed-phase adsorption chromatography on a second column before analyzing the extract by HPLC. Figure 4 shows some typical results. As can be seen, chromatograms B and C are much cleaner than A which was without cleanup. The same procedure was also used to analyze human serum from a number of people hospitalized due to domoic acid intoxication. The chromatograms looked very good and in some cases a peak indicative of domoic acid was found (Figure 5). However, when the samples were carried through the additional cleanup procedure as used for the urine samples, no peak was observed for domoic acid. The original peak was, therefore, not domoic acid.

Several chemical confirmatory tests have been developed for domoic acid. They involve forming highly fluorescent or UV absorbing derivatives with different chromatographic and detector characteristics than the parent compound. The fluorescent derivative method using fluorenylmethyl chloroformate (FMOC) was developed by Dr. Quilliam of the National Research Council in Halifax. The derivative is extremely fluorescent and can be detected by HPLC in low picogram quantities. For those analysts who do not have a fluorescence detector, UV absorbing derivatives employing phenylisothiocyanate or acid/isopropanol can be useful[5]. Figure 6 shows a confirmation using phenylisothiocyanate, while Figure 7 shows a similar confirmation using acid/isopropanol. As low as 5 ppm domoic acid can be confirmed by these procedures.

In conclusion, domoic acid can be easily determined in shellfish by direct HPLC with UV detection at levels greater than 1 ppm. The same method is also useful for feces samples. For urine and other biological fluids, additional sample cleanup is required. Determination of domoic acid at levels substantially below 1 ppm requires additional sample concentration, cleanup or chemical derivatization.

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9. *Mussel Culture and the Accumulation of Domoic Acid*

G.R. Johnson, L. Hanic, I. Judson, P. Nijjar, and A. Tasker

Abstract

The cultured mussel industry of Prince Edward Island had never experienced a toxic disease problem until November of 1987. With the successful use of the long-line culture system, the yearly production of fresh mussels to the gourmet food market had risen to close to 3.2 million pounds (1.46 million kg) of product. The physiology of this sessile bivalve and its method of feeding and location in the estuary leave it prone to the accumulation of a widely distributed biotoxin. Eastern Prince Edward Island became the epicentre of domoic acid-intoxicated mussels as early as 10 November 1987 (retrospective samples) during an intense bloom of the diatom *Nitzschia*. Mussels were able to accumulate large amounts of the domoic acid with little effect on their own well-being. Despite being in low water temperatures (below 4°C) and under thick ice cover, the levels of the toxin decreased and were undetectable in about 6 weeks. The following year the toxin was detected in much smaller amounts, and the levels of toxin accumulation demonstrated a variable lag time with the increase in concentration of *Nitzschia* available in the water column. The sales of Prince Edward Island cultured mussels have rebounded to about 140% of the pre-domoic acid crisis.

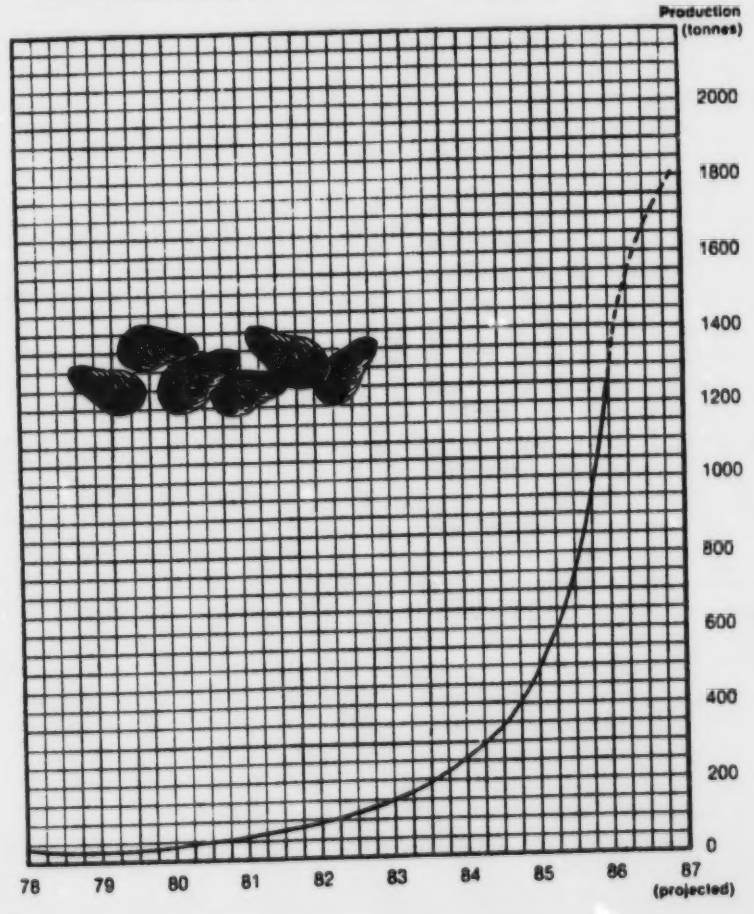
Introduction

The cultured mussel industry on Prince Edward Island is a dynamic and growing form of aquaculture, supplying, fresh to the market, a gourmet product. This paper describes how the industry produces mussels, where the domoic acid problem occurred, how these bivalves accumulate toxins and what happened to the domoic acid levels subsequent to that devastating episode of intoxication in 1987-88.

The cultured-mussel industry

Beginning with the most basic of experimentation in 1975, the mussel industry continued to grow exponentially through the 1980s. Characterized by ingenious innovation and aggressive marketing, driven almost entirely by entrepreneurs, the industry harvested up to 3.2 million pounds (1.46 million kg) of product in 1988 providing the lion's share of the cultured mussels from Atlantic Canada (Figure 1). There appeared to be no market limitations. Since there had been no history of paralytic shellfish poisoning on eastern Prince Edward Island, no consistent monitoring program was in place.

Figure 1
Prince Edward Island's Cultured Mussel Production, 1978-1987



The culture system, while not entirely unique, underwent numerous adaptations to evolve into the highly successful long-line system that is used today[1]. Life for a mussel begins with the release into the seawater of sperm and eggs from dioecious adult mussels. There are a few motile stages that occur within weeks and then a miniature mussel evolves to attach to almost anything available[2]. Long lines are heavy nylon ropes anchored by moorings at each end and suspended in the water column with buoys of various sizes. Polypropylene ropes, new or previously used in the lobster fishery, suspended on these long lines, collect the spat which are

harvested in September and "socked." Socking consists of collecting spat and loading it into nylon mesh used for containing mussels until they attach their byssal threads. Eight-foot (2.7 m) socks filled with 3/4 inch (1.91 cm) mussel spat at 175 and 200 per linear foot (1/3 m) are suspended from the long line. Predators, such as starfish and crabs, cannot easily gain access.

During the winter months, the long lines are sunk with sandbags so they remain unaffected by ice damage. Harvesting through the ice is a Prince Edward Island specialty that allows the mussels to be cut from the long line, transported to the processing plant, cleaned, debyssed, packed, and put on the restaurant table in any major North American city within 2 to 6 days.

Mussel physiology

The mussels migrate to the outside of the sock, opening their valves to filter the algae in the passing sea water. A resident time of 12 to 18 months in the sock produces a 50 mm to 70 mm market mussel. In estuaries that are less productive, mussels may remain on the socks for up to 2 years. Cultured mussels have no opportunity to manufacture the "pearl" (characteristic grit often noted by consumers of wild mussels) as they grow quickly and are marketed before the 3 1/2 years required to produce noticeable pearl formation.

Mussels cultured in this fashion utilize the biologic capability of this bivalve to the maximum. Mussels are osmo-conformers, wasting no energy compensating for changes in their environment[3]. They are ectotherms, making no attempt to maintain a specific body temperature. The feeding response is controlled by algal density, and they are not fussy about size of particles consumed — anything from 2 to 90 microns. They are truly sedentary and accept their position in growing slowly in a poor environment, but when exalted to the best place in the water column, they feed voraciously, siphoning 2 to 5 L/h and extracting up to 98% of the available algae[3]. The rapid growth produces thin broad shells and plump meats with meat-to-shell ratios in excess of 45%[4]. The fatty acid make-up and the cold harvesting provides a sweet delicacy with optimal nutritional value[5].

The geographic location

The estuaries most heavily farmed on Prince Edward Island are located on the east end of the Island. Most suitable water is leased. The estuaries are chosen because of reasonable tidal flush, protection from adverse weather, limited ice movement, and appropriate depth (25 to 40 feet [8 to 13 m]). These particular estuaries are surrounded by cottages and farm land and have relatively busy transportation rights-of-way.

The earliest sample we have of toxic mussels was 10 November 1987, from St. Mary's Bay, a sample taken out for other research and submitted for analysis retrospectively. About that time, both of the large export plants switched much of their harvest to the adjacent Cardigan River. Those mussels appeared on the market simultaneously to the first recorded human illnesses. By 1 December, the Prince Edward Island mussel industry was shut down with its first disaster ever. By 17 December, Atlantic Regional Laboratory of the National Research Council in Halifax had isolated the offending toxin, domoic acid, an amino acid neurotoxin, and demonstrated that this compound produced, in mice, identical clinical scenario as extracts of toxic mussels[6]. The mouse bioassay used for paralytic shellfish poisoning was modified as an expedient screen for toxicity and the high performance liquid chromatography method was used for isolation and confirmation of exact amounts. Mouse bioassay has subsequently been refined at the Atlantic Veterinary College to quantitate low level responses as well as seizures and death (A Tasker: personal communication, 1989). Concentrations of 20 mg/L can be reliably detected by bioassay. This allows detailed study of the behaviorial pharmacology of domoic acid.

During the chemical search for domoic acid, investigators in the field had found that over 90% of the algae available to these mussels was *Nitzschia*. Whether 6 miles (10 km) offshore or in the estuary, there was domoic acid associated with *Nitzschia*[7]. The mussels in this case were not sick, they were thriving. They consumed the *Nitzschia*, as demonstrated histologically[8]. As they did so, they accumulated eosinophilic intracytoplasmic droplets in large numbers. Cell size increased immensely. The droplets were green on toluidine blue staining of thick plastic sections and electron dense, nonmembrane bound on EM. They appeared morphologically to be a phospholipid[9]. Whether or not they contain domoic acid has not, to my knowledge, been determined. They may accumulate due to inhibition by domoic acid of normal metabolism.

Excretion or consumption of droplets coincides with a drop in domoic acid level in the tissue, though there is a variable lag time rather than a line or correlation of chemical decline and morphologic decrease in droplets (L. Hanic: personal communication, 1989). In the Cardigan river, even at less than 4°C, under the ice, the levels of domoic acid in tissue decreased relatively rapidly and were gone in less than 6 weeks.

In the fall and winter of 1988-89, the curve of toxicity paralleled the curve of *Nitzschia* concentrations in the water column with a lag time of up to 10 days depending on the estuary (J. Worms: personal communication, 1989). Levels of *Nitzschia* or domoic acid were much lower than the previous year. The rivers were sampled and monitored by Fisheries and Oceans in 1988, and rivers were closed when levels approached 20 mg/L (J. Worms: personal communication, 1989).

Following re-entry to the market, consumer acceptance rebounded quickly with 1988 sales being 140% over 1987 sales. Based on the actual months mussels were marketed in each of those years, the average monthly shipment was 46% over 1987. The market is sensitized to media coverage of any aspect of the toxicity phenomenon; sales drop overnight by 40% to 50% of the current level at any announcement and gradually rebuild to even higher levels. Lower prices, however, have meant that producers have not reaped the benefits of increased markets.

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Discussion: Presentations 7, 8, and 9

Question from the floor: Dr. Lawrence: I am just wondering with regard to these 2 column cleanups that you use on the urine samples and the serum samples, if you can give us some indication as to how much that decreases the recovery.

J. Lawrence: We find, I think, by about 15% to 20%. In other words, if you take domoic acid through the cleanup, you'd recover about 80% to 85%, which means from the initial extraction, if you are extracting only 75% you'd end with about 60%. You can actually play around with that and improve the recoveries. I think Mike Quilliam at the National Research Council was getting, with a type of cleanup which is very similar, up to about 90% or more through the additional cleanup.

H. Conacher: Dr. Wright: You mentioned that *Nitzschia* was the source of domoic acid. Can you also get kainic acid in *Nitzschia*?

J. Wright: No, we have looked very hard but we haven't seen that. Actually, I want to back up a couple of paces in that the culture of *Nitzschia pungens* that we have is not bacteria free. So, there is still a possibility that it could be a bacterium associated with *Nitzschia pungens* responsible for making domoic acid. That is still a possibility. But it turns out that there are a number of glutamate agonists like kainic and domoic acid that are produced by algae. I don't know why that is, but it is rather interesting. In fact, a very common red alga called *Palmaria palmata* dulse which is a common delicacy for the Japanese actually has the ability to make kainic acid. It doesn't make it in the wild type but we have isolated some spontaneous morphological mutants of *Palmaria* which make it. So the feeling is that kainic acid and domoic acid may be some throw back in evolution, that the genes are obviously there to make it in *Palmaria* but they are not normally expressed and only in certain mutants do you get the expression for the enzymes that will make kainic acid. I wanted to also mention that Frank Iverson talked about the use of kainic acid as anthelmintic in Japan. It is actually very common in China as well. We have a native Chinese scientist in our laboratory and when he went back home we asked him to bring some of those powders back which he did. The alga that they use in China as an anthelmintic is called *Digenia simplex*, and the powder should contain kainic acid. This is less potent than domoic acid but we couldn't find a trace of kainic acid in the powder. So, obviously China's equivalent to our Department of Corporate and Consumer Affairs doesn't work so well, because they are being told that this is an anthelmintic but it didn't have any active ingredients, i.e., kainic or domoic acid. Apparently it depends on the season when the *Digenia* is harvested because it is a seasonal production.

A. Novelli: Just for curiosity, Dr. Wright: you speculated about the possibility of the repression of a gene for the production of kainic acid and domoic acid in the algae. Do you think there may be a viral infection of *Nitzschia pungens* instead of a bacterial one?

J. Wright: That's a possibility. We have done a fair bit of EM work but I can't say that we have ruled out any viral infection of *Nitzschia.* I am trying to remember the results of that. I don't think any viruses were found, but I shouldn't even comment on that. That is a possibility.

10. Panel Discussion: Evidence that Domoic Acid was the Cause of the 1987 Outbreak

I. Hynie, J. Hockin, J. Wright, and F. Iverson

I. Hynie: The evidence that domoic acid was the cause of the 1987 outbreak of toxic mussels poisoning is quite overwhelming and as it has been discussed here by different speakers in considerable detail, I will review it only briefly:

- All mussels eaten by patients included in the outbreak were traced to 2 leases in the Cardigan Bay, Prince Edward Island.
- The mussels ("toxic mussels") in these 2 leases had the highest content of domoic acid among all Prince Edward Island mussels tested.
- All toxic symptoms in rats and mice caused by extracts from toxic mussels could be qualitatively and quantitatively reproduced by spiking non-toxic extracts from normal mussels with equivalent amount of domoic acid.
- No fraction of different extracts from toxic mussels other than that containing domoic acid had toxic properties in animal experiments.
- The human syndrome is extremely variable but all individual clinical symptoms of human illness are compatible with experimental findings of studies with ∝kainic and/or domoic acid.
- Neuropathology of 4 patients who died in the outbreak was specific and strikingly similar to the findings in experimental animals poisoned by domoic acid.

Our task is now to compare this evidence with a few problems which cannot be ignored:

1. The standard epidemiological procedure is to identify suspect cases by certain set of criteria and verify them by a laboratory test. We did not know what to look for during the outbreak and no urine or feces were available later. Consequently, domoic acid was never identified in human material.
2. Our case definition restricted suspect cases to those who ate Prince Edward Island mussels. Consequently, eating Prince Edward Island mussels cannot be used as evidence that nothing else could have been involved.
3. Human cases ingested less than 10 mg of domoic acid per kg of body weight, some probably as little as 1-2 mg/kg. While these doses are highly toxic to mice if injected parenterally, both mice and rats can tolerate 50 mg/kg orally without any toxic effect.
4. For each symptomatic case there were about 500 people who ate toxic mussels without any ill effect. It seems that even some of the most severe cases followed a meal which was quite harmless to other diners at the same restaurant table.
5. Domoic acid-rich extracts from algae have been used in Japan as home remedies against certain parasitic diseases for centuries. Even purified domoic acid has been tested there on children (6-20 mg total dose) with no ill effects.

As eating of domoic acid-rich mussels was part of the case definition, I'd like to address my first question to Dr. Hockin: Were there any patients identified who were later rejected because they didn't eat mussels?

J. Hockin: Yes, this raises the major bias in the kind of information we were collecting on cases in that our case definition was restricted to people who ate Prince Edward Island mussels. We made up our case definition for the purpose of identifying additional people who were ill early on in December 1987, and we wanted to make it fairly restrictive because it included the possibility of a case of someone being identified as a case of illness related to this outbreak because he had minor gastrointestinal symptoms within 24 hours of eating mussels. We, in fact, thought we would be flooded with case reports of people who had eaten mussels and other kinds of shellfish. In fact, we did get reports at L.C.D.C. and the local people got reports of illness related to all kinds of products but it was a fairly small number of cases and there were no severely ill people who had eaten other products. That is to say there were no people who seemed to have the neurologic syndrome who had eaten anything but Prince Edward Island mussels. I think if we restrict our thinking to those severely ill, we have so many cases who ate Prince Edward Island mussels and we have zero cases of people who ate all kinds of other things over the same time period. So there was no need to do any further epidemiological investigation of that relationship case to non-case.

I. Hynie: Would it be fair to say then that the neurological cases, or patients with neurological symptoms were all associated with toxic mussels, while patients with only gastrointestinal symptoms may or may not have all been properly identified and we will never know?

J. Hockin: Well, the gastrointestinal symptoms we included in the case definition are quite nonspecific. This could be caused by a myriad of things, including other shellfish toxins, bacteria, viruses, food allergies and so on, and we suspect that we may easily have included some of those amongst our cases had we left the case definition wide open to eating any shellfish. That wasn't the case with the neurologic illness and if there is anyone who knows of people who have memory loss after eating something other than these mussels under the same circumstances we would like to know about it.

I. Hynie: The other side of the coin is, of course, the fact that certain regions were probably under-reporting. One such region was the United States where there were no reports of cases and yet from the source figures of toxic mussels shipments one would have to expect a certain number of cases there which were simply not identified. If we accept something between 1 and 3 cases per 1,000 servings, then in the United States you would have expected somewhere between 10 and 20 cases, I believe. So,

the numbers may be biased downwards rather than upwards.

J. Hockin: Yes, there is quite likely under-reporting even of severe illness. Based on the sales figures that Gerry Johnson put together in terms of volume sold there, we would have expected between 15 and 20 cases to be reported from the United States if their surveillance system was as good as that in the province of Quebec where we saw approximately 2.2 cases per 1,000 portions of mussels shipped out of Prince Edward Island. That ratio of cases to mussels shipped was somewhat lower in the province of Ontario which got about the same amount of mussels as did the United States and we had a small number of cases reported in Ontario. The fact that there are not any from the United States and the fact that there were relatively few cases from Ontario can, I think, be attributed to case finding that wasn't as diligent as in the province of Quebec and the Maritime provinces.

I. Hynie: Let us assume that the link between mussels from a particular area and the disease can be considered as solid as is possible under the circumstances. The group in Halifax isolated domoic acid out of these mussels and I don't think there is any question that what you have isolated is domoic acid. The question which may be asked is, could you have missed something else? You have pretty well reviewed that already but would you mind briefly reviewing not what evidence you have that the domoic acid was there, but rather what evidence you have that nothing else was there.

J. Wright: There is certainly nothing else that we have found so far. We have obviously looked at that very hard. We looked at that in December when we had an active fraction that was toxic and we were zeroing in on domoic acid and obviously we wanted to be sure that domoic was the only compound that was giving any problem. We looked at the beginning of the chromatogram, everything that eluted from the column from time 0, right up to the end of the chromatography experiment and even at what we call heart-cutting the peak of domoic acid, really cutting it right down into the base of the peak, and we could find nothing - no activity outside of the domoic acid peak. When we went back in January and constructed a much more sophisticated time of death curve and compared pure domoic acid with mussel extracts and with control mussels and did various types of extractions on mussels, the time of death curve was almost identical for every experiment, whether it would be pure domoic or whether it would be shellfish extracts — extracted with water or with acid. We feel very confident that we have proven that domoic acid is responsible for the toxicity. The work that we are doing now with domoic acid isomers would suggest that yes, there is peripheral toxicity arising from the original domoic acid peak in the mussel but that toxicity is not significant, a) because these compounds are there in about 1% to about 8% compared with total domoic acid concentration and b) they are also at least one order of magnitude less toxic. So, we are pretty confident that domoic acid is indeed responsible for the toxicity in the mussels.

I. Hynie: You are talking of course about mouse model, IP, and about being reasonably certain that there is nothing else in the mussels which can poison mice if injected parenterally.

J. Wright: Sure, we spent a few days before we got into the water soluble compounds looking at the organic or lipid soluble compounds and we couldn't kill mice with anything in the lipid soluble fractions, and we sent you and Todd fractions that were really loaded and we loaded the dice in our favor to try and kill some mice and we couldn't. I do want to mention that we found a completely different pigment profile in the control mussels and the toxic mussels in the digestive glands. Not only do they look different, and Gerry Johnston eluded to that fact, but they also smelled different. In fact, they are different and it just certainly reflects the diet that they are eating.

I. Hynie: Now, we will proceed to the next question. The problem as voiced by some people, if I understand it correctly, is first of all, the Japanese claim that domoic acid is not toxic to people. It is difficult to find data in literature from Japan on what experiments they have really done in humans. There are a number of data on their experiments in mice and rats. These are pretty well in line with what you have heard here today. As far as their human experimentation goes the only thing we were able to find out, and perhaps some people here may have been able to extract some more information, was that they had 3 trials: twice they have given 6 to 8 mg total dose to a group of about 10 to 15 children. We don't know their ages. In one experiment they have given 20 mg total dose and they were apparently satisfied that there were no side effects. They also claim that for hundreds of years their fisherman were eating extract of seaweeds to treat different intestinal parasitic diseases, seaweed containing either kainic acid or domoic acid and that this remedy was not toxic. The problem is that, of course, if you know that the extract from the seaweed is not toxic, then if a fisherman in some village dies you know it is not from the seaweed because that stuff isn't toxic so it must have been something else. So, you are essentially perpetuating this notion that it is not toxic. We cannot, however, ignore the fact that to poison a rat or mouse by an oral dose of either toxic mussels or domoic acid orally is almost impossible or at least difficult. We don't have any data on humans and the only thing which sort of mitigates for the acceptance are the monkey experiments which do indicate that the oral dose is sufficient. Frank, would you like to comment on the oral versus parenteral dose and the inter-species differences?

F. Iverson: Well I don't know how much I can add, it is summarized pretty well there. But yes, our results were pretty much the same as far as the acute IP studies that Jeff Wright has covered. We certainly didn't find anything else other than domoic acid. Let's put it this way, domoic acid in the concentration in which it was in the toxic mussels certainly accounted for all of the symptoms that we saw. As far as the Japanese studies go I can make perhaps several comments: I don't have more data than you have, but I think we have already heard that we certainly wouldn't know how much domoic acid was present in these home remedies they have taken. So, really all I can comment on is the one study where apparently they gave 20 mg doses to children. This works out, depending on the weight, I think to about 0.3 to about 0.7 mg per kilogram. In our primate studies if we gave about 0.5 mg we didn't get any effect. As best as we can determine it

seems that in some of the individual human cases they could have easily taken 10 times that amount. So, it is a question of the amount eaten and I think the amount of domoic acid was there.

I. Hynie: I believe Dr. Perl was quoting some numbers which started with a total dose of something like 50 mg in adults which would be actually less than 1 mg per kilogram and it was already toxic at that point which would mean that the Japanese may have come very close to a toxic dose if you are right.

Question from the floor: From our mice data it is quite common to see a 4 to 5-fold difference in the dose before you see a response. So, it is not difficult to imagine that humans would have an equal amount of variability. It is the aging effect which is what I thought was most informative about the human cases and there is good evidence that there is an increased susceptibility to toxins which has been demonstrated in animals, particularly for the hippocampus with aging, and you get a natural cell death that occurs in animals in exactly those areas that are aging affected particularly by kainic acid.

F. Iverson: Yes, the age effect certainly is important and I think we can get to that a little bit later. I think one of the problems that arises here is that some of the Japanese data refers to rodents and I think we know, at least from some of the data that I have presented here this morning, that the rodent just doesn't absorb it at all if it is given orally and that it is a poor choice of species for extrapolating these data to humans. I caution again that we are not finished with our studies yet. We have only been through once with the primate and we can see effects where we wouldn't begin to see them with the rodents at about the same dose that at least some individuals were consuming. There is absorption and it is probably an absorption phenomenon. So it's a species difference and I would suspect that we will see more of this as we get more data and this is probably one of the reasons. I cluded to 3, 4 or 5 different reasons why people might be predisposed to this this morning, and I don't think there is anything in here that indicates anything else other than domoic acid being a toxic compound. Absorption with something else could be explored or perhaps a hypothesis could be built to say that there is a possibility that something else may aid in the absorption or something else such as another amino acid may increase toxicity, perhaps. These are all things for which we have no particular data at the present time but nothing indicates that there is anything other than domoic acid that is giving the effect that we would expect.

I. Hynie: Now if I may go to another topic: I claimed that people who are sick have certain symptoms that I believe can be compared to the results of the animal experiments and I would ask Dr. Teitelbaum if she can at least comment on what she has witnessed herself as neurological symptoms in people in comparison with the results observed in animal experiments. I don't think that there was too much on the monkeys, unfortunately, but at least one sees a much lower level of dose required.

J. Teitelbaum: Probably the most similarity is with the convulsions and the abnormal movements that we saw in human beings in that they had chewing movements, myoclonus, and seizures: all things that are reported in rodents and animals that have received kainic acid or domoic acid. As far as the memory abnormalities are concerned we have no comparison right now in the animal and studies will have to be done looking at learning in the animals before we can answer that question. Many people have brought up the fact that it is possible that what we found in the human beings had nothing to do with domoic acid *per se* and that just the patients were acutely ill, hypotensive, and the hippocampus is very sensitive to ischemia and maybe all the memory changes that were seen, the hippocampal damage, has to do with that. I think it is unlikely. I think I did show in some of the patients that they never had any hypotension and had no other risk factors that would explain the incredible memory loss that we found and that is about the only argument that we have at the present time. The other question is the motor axonopathy. There is some evidence in the literature that motoneurons are affected by kainic acid and by domoic acid and this area in the spinal cord is relatively resistant to ischemic damage and so the arguments about ischemia wouldn't pertain to the spinal cord.

I. Hynie: I have also mentioned the similarity in the neuropathological postmortem findings in man and animals. Now, I didn't invite Dr. Carpenter up here, for the simple reason that he will be presenting his paper tomorrow, so he didn't have a chance yet to say what he has to say. I would just like him to comment briefly, whether what I am saying today and what he is going to say tomorrow is mutually compatible.

S. Carpenter: First, I should say that what I saw in all 4 cases was very stereotyped so that these 4 patients who had died after the mussel ingestion at various time intervals all seem to have the same lesions, virtually identical, though slightly less in one person. Now secondly what else could they be? One brings up the possibility of hypotension and ischemia. This was certainly present in at least 2 of them and probably 3 of them did have hypotension but not early in their course. If it had been hypotension one would expect hippocampal damage — yes. One would not expect the amygdala to be severely damaged as it is; nor would you get lesions in a claustrum and in this region inferior to the basal ganglia — let's call it olfactory tubercle area — I wouldn't expect that in ischemia at all so it seems to me ischemia is not a really good explanation for what we see. I can't think of anything else. Now if we compare this to what has been shown with administration of kainic acid to animals the lesions are very, very similar. The pattern in the hippocampus, I think, is quite similar to what has been shown with kainic acid given systemically. If you put kainic acid into the ventricle you get lesions particularly in CA3 and I didn't see that. The damage to the hippocampus — it damages all the hippocampus with some relative sparing to CA2. On the other hand, when you give systemic kainic acid, according to what I have read, if you look at the animal at 1 day CA3 is the most damaged but if you look at the animal after 3 days and later on the damage in CA1 is at least as extensive as in CA3. So, this is quite consistent with what I see. So, lesions are in the same area as in kainic acid given systemically and I don't know what else would account for them.

I. Hynie: If I may ask a question at large. Does anybody here feel that first of all there was something which we have forgotten to look at as a possible

proof that it was domoic acid, or secondly, that there is an alternative explanation for all that and that in fact it may not be domoic acid?

A. Novelli: Well, I think that ischemia may have even worsened in this situation induced by domoic acid so that the presence of domoic acid plus the presence of an ischemic situation could have made things worse in non-healthy people.

Question from the floor: I hate to show my ignorance in chemistry but would there be the same proportion of isomers of domoic acid in all sources including the synthetic domoic acid available?

J. Wright: No, it seems to vary depending on the source of the domoic acid or at least the matrix the domoic acid is in. I did show a slide where you can see traces of the isomer in cultured phytoplankton (laboratory culture). You see a little bit more of them in phytoplankton harvested from the Cardigan Bay and you see even more of them in the mussel tissue whether the mussel tissue has been cooked or not. We have actually synthesized these isomers now in the laboratory because we got tired of extracting kilos and kilos of mussels to try and get a few micrograms of isomer and we have a good idea now how they are made in nature, and it's not an unreasonable process to imagine how they might be generated in nature from domoic acid.

Question from the floor: If it is the receptor mediated toxicity, as we think excitatory amino acid receptors, that is going to be terribly important, and it may account for differences in what you are measuring in terms of potency or activity. When the isomer profile is different, you are working with different amounts of the active substance.

J. Wright: Yes, that is true. We obviously were concerned about that and we were even more concerned about the fact that maybe the isomers may be more toxic than the domoic acid itself. The work that John McDonald has done and the work that David Hampson has done which I didn't mention and which incidentally is in parallel with McDonald's findings, do seem to suggest that the geometrical isomers at least are much less toxic than domoic acid itself. But let me re-emphasize again that we are talking subtle differences here and that isomer profiles compare from plankton to mussels. Even in the worse case when they are "the most obvious" they still amount to less than 10% of the total amount of domoic acid in the shellfish extract and that is in the worst possible case. More likely when you look at them it is somewhere between 1% and 5% of the total.

Question from the floor: I wanted to bring out another point. How would you explain the delay between the ingestion of the toxic mussel and the appearence of symptoms especially the neurologic symptoms compared to what you have seen in the rodent or the primate experiment?

I. Hynie: I believe that as far as the delay in ingestion and the gastrointestinal symptoms this is something which will be addressed tomorrow and was addressed to some extent today: it seems that, in fact, gastrointestinal symptoms are not directly caused by domoic acid locally, but are induced through the central nervous system. The delay could be that you have very slow transfer of the poison through the blood-brain-barrier so it may take some time before sufficient concentration is developed in the brain to become effective. Obviously, in rodents it would be faster than in people. But we really don't know very much about these processes yet because we know next to nothing about metabolism of domoic acid in any animal, including humans, and we don't know very much about its transport across the blood-brain-barrier either.

Question from the floor: Obviously a lot more people ate mussels than got sick. Have you got an explanation for that?

I. Hynie: I think that this is something which has already been commented upon here. The numbers are roughly something like 500 people eating mussles not getting sick to 1 that does get sick. It would be a combination of probably 3 things. One would be the dose, the other one would be the state of health, including age, and the third one could be genetic predisposition. One can add factors which we really know very little about such as what was eaten with the domoic acid, or what was eaten before the domoic acid. For example, a high protein meal, high intake of glutamate, may improve or decrease your chances of getting sick. But the only hard fact we have is that most people who did get seriously ill were people who already had some underlying condition such as renal diseases, liver failure, cardiac failure, atherosclerosis, or diabetes. There are some people who did become sick who were quite healthy before. So, it is again not so absolute. If there are no more questions, I'd like to thank the members of the panel and ask Dr. Perl to take over for the next discussion.

11. Panel Discussion: Definition of the Syndrome

T.M. Perl, J. Teitelbaum, J. Hockin, and E.C.D. Todd

T. Perl: The question that we were asked to address is the definition of the syndrome and I thought that there were several aspects to this. One would be the clinical definition and the second part would certainly be the laboratory aspects of the definition that would be necessary. What I have done is put up the epidemiological definition that we used as a starting point and I realize that there will be problems. For example, we would probably not want to have in the definition "mussels from Prince Edward Island" and I thought that we could start from here. Some of the biological scientists and perhaps some of the clinical people here, probably just Jeannie Teitelbaum and I, may want to comment on the gastrointestinal aspects to start. I know that, in terms of the epidemiology, we saw a wide variety; they were nonspecific, and they probably would be difficult to use as an identifier of the syndrome. However, what may be part of the syndrome would be the duration of the symptoms.

Although we don't have good information, we do have some evidence to suggest that these did persist, instead of just 1 or 2 days, at times, up to a week. Does anyone have any feelings about that?

J. Teitelbaum: I am not sure what you mean by duration — perhaps acute symptoms and just the gastrointestinal?

T. Perl: Well, from the period of onset until the disappearance of the symptoms, i.e., the patient may present with nausea and vomiting and that may persist for up to a week or for 10 days.

J. Teitelbaum: Certainly I only saw a small group of patients and these were the sickest ones, but the vomiting which started anywhere from 1 hour to 24 hours later didn't persist more than 72 hours in any of the 14 patients in this group. The gastrointestinal bleeding that did occur was sometimes much, much later and was thought, at the time, not to be related to the domoic acid itself. This may be wrong, judging from experimental studies. Looking at that again, 2 people did present with gastrointestinal bleeding within that 24-hour range, and 1 person, up to 3 months later. This action might be part of the syndrome if there are ulcerations in the gastrointestinal tract because of domoic acid. There may not be clinical bleeding until much later if it is subclinical. I don't believe that we looked for occult blood in these patients, at least those who were hospitalized in my hospital.

T. Perl: Is there anybody from Winnipeg, Dr. Pinsky's group, who would like to comment on how characteristic the findings were? I know you will talk about it tomorrow, but in general, do you think they are characteristic enough to be included?

G. Glavin: Yes. I can comment on the fact that Dr. Teitelbaum mentioned. One patient at autopsy indicated a duodenal lesion — almost a perforating duodenal lesion. I will mention tomorrow that both the whole mussel extract as well as pure domoic acid makes very nice duodenal lesions in rats which is very hard to do. Those of us who are in the business of gastrointestinal research know that it is hard to make a duodenal lesion in a rat. It is very, very difficult to do with unphysiologic doses of nicotine and proprionitrol and all kinds of unspeakable things. Here we have very low doses and very modest amounts. So, I would say that the duodenal pathology is very, very typical and the rapidity with which we see the gastrointestinal bleeding and hyperemia in mice within 15 to 20 minutes is also atypical and hard to get. So, I think that argues for the inclusion of that. Although what argues against that, I guess, is the number of patients who, by your criteria, had the syndrome but didn't have any apparent gastrointestinal problems and that is a puzzle.

T. Perl: Well, we didn't look. This is part of the problem.

Question from the floor: You mentioned about the excessive secretion or pulmonary edema. Is that more characteristic even than the vomiting and the diarrhea? That is a real common effect known to anyone who has worked with kainic acid lesions in animals. A number of them die as a result of this effect.

J. Teitelbaum: Looking at the 14 patients who were the more severely ill, at least 50% of those showed increased bronchial secretions which made us look for organophosphates. Actually, I don't know that it is more important than the nausea and vomiting which affected everyone except for perhaps 1 or 2 patients. Maybe it is proportional to the severity of the intoxication.

Question from the floor: What about hemodynamic irregularities?

J. Teitelbaum: We really think there was autonomic cell damage and the fact that so many patients showed brady and tachyarrhythmias and hypo- and hypertension as well as bronchial secretions — all favor that. We haven't proven it though. Maybe we could move to the neurologic and then we could discuss the other symptoms which include the cardiovascular and the autonomic.

I. Hynie: Just wondering whether apart from a general definition of the syndrome we may also need a more detailed description which could be useful in diagnosis of sporadic cases. The 1987 outbreak will probably never happen again but a possibility of sporadic cases is there. The most common symptoms, such as the gastrointestinal ones, are also very nonspecific and not too helpful in a sporadic case. Symptoms such as bronchial secretion, blood pressure irregularities, and all the neurological symptoms may be less common, but they are more specific. I also feel that diagnosis of a sporadic case will have to be confirmed by a laboratory test, preferably in feces or urine. Perhaps you may find it more profitable to discuss the broad description of the syndrome today and try to reach a consensus on the definition in a small group later.

J. Hockin: In response to that I will tell you my bias. I look at this syndrome as parallel to something like paralytic shellfish poisoning which is clearly a gastrointestinal illness, whether it is centrally or locally mediated, which has other manifestations in some people. It is these other manifestations which define the syndrome as domoic acid poisoning

and not something else. In sporadic cases of mild illness, because of the potential for severe illness, I think it is very important to identify the gastrointestinal illness as being potentially domoic acid caused, and I think the best way to do that is to collect a nonclinical specimen, such as mussels, for which the methodology is very well defined. I would suggest that mussels or molluscs of other species are the specimen of first choice in trying to make a clinical diagnosis. Not to the exclusion of trying to develop an assay which may prove important in rapid diagnosis of someone presenting with severe illness. I would like to place the emphasis on surveillance and differentiation at an early stage of mild cases rather than very severe.

Question from the floor: You mentioned other molluscs. Did anyone find other bivalve molluscs such as oysters, and clams, etc. besides mussels that had tissue levels greater than 20 µg/g of domoic acid?

J. Wright: Yes, there were quite a lot of historical samples taken and there was also a survey done by Fisheries and Oceans sometimes in collaboration with us and sometimes on their own. Correct me if I am wrong but, as far as I know, the only other shellfish that has been found to contain levels of domoic acid at all, but certainly over 20, has been scallops and only in a digestive gland, and since the digestive gland isn't eaten in scallops then it is not a problem as I understand it.

Comment from the floor: Just a comment on that. We have found it in the soft shell clam over that level and in the red mussel and it looks like it is fairly prevalent among the filter feeders that live along the shoreline. The scallops are, at least, in deep water. We didn't find it in some of the other hard shell clams that are in deep water for whatever reason.

C. Pinsky: I would like to ask Dr. Teitelbaum and Dr. Perl if there were any electroencephalograms taken early in any of the syndromes. Also, is there a rapid test for visual spacial deficits and how early did these show up in the syndrome?

J. Teitelbaum: The EEG was done early, as early as 48 hours after ingestion, in about 3 of the patients — 2 of them at the Royal Victoria Hospital — and they showed at that time severe generalized disturbance of activity with slow waves throughout. It seemed to be more predominant in the left hemisphere than the right, but not strictly localized. Within 24 hours after that EEG, one patient showed generalized epileptiform activity, at times coming from the left temporal lobe, at other times being focal motor left hemisphere, and at other times being generalized.

J. Teitelbaum: The digestive upset started before the EEGs were done and were almost resolved by the time the EEGs were completed, only when they became confused about 24 hours later. Visuospatial testing was done, formally for the first time, about 4 months after, except for one case at Notre Dame Hospital, which may have been done earlier.

T. Perl: I would be concerned about the practicality of doing visuospatial testing in a severely ill patient. I am not sure that would be very sensitive and it just may not be practical.

Question from the floor: This morning in your presentation you had come up with the possible title of amnesic shellfish poisoning as the title for this symptomatology and this afternoon we have just heard Dr. Hockin talk about it as a gastrointestinal syndrome. I am wondering if we could get a better handle on really what we are talking about. There seems to be quite a divergence in the 2 titles for this syndrome.

Dr. Hockin: I may be responsible for the term amnesic shellfish poisoning too. I called paralytic shellfish poisoning a gastrointestinal syndrome as well. My rationale for amnesic shellfish poisoning was that it seemed to be the cardinal neurologic effect which parallels the paralytic effect of paralytic shellfish poisoning also. Amnesic shellfish poisoning was open for use and some of the other possibilities didn't seem to be. I think, in light of what Dr. Teitelbaum has presented, amnesic is a fairly broad term but seems to fit with the permanent loss suffered by some people.

R. Auer: I would like to know clinically how low did the blood pressure go in the patients that didn't have atherogenic heart disease?

J. Teitelbaum: The 3 patients who were most severely affected and who went into shock had blood pressures around 90 systolic and untraceable diastolic. In one patient there was atherosclerotic heart disease but that didn't seem to explain the problem. These patients were in intensive care where they had Swan-Ganz catheters and would seem to have a sudden massive vasodilatation for no logical reason. They were given tremendous amounts of fluids which corrected the blood pressure but then this vasodilatation, this drop in resistance, reversed itself on its own — no treatment. They subsequently went into pulmonary edema and we had to get rid of the fluid we had given them. Our only explanation was that it was autonomic. There was no cardiogenic problem at that time in most of these patients and it really seemed to be an autonomic vasodilatation.

R. Auer: I guess Dr. Carpenter will show us tomorrow if there were parasagital lesions suggestive of ischemia, but I am also skeptical of the proposed synergism between ischemia and excitotoxic lesions. Certainly, there is no precedent for such a situation in the literature experimentally, and if you lower the blood pressure in various excitotoxic situations you merely shut off the epilepsy but you do not exacerbate the brain damage.

J. Teitelbaum: It is not even clear that the brain damage would be in the same place with ischemia and with domoic acid but we would like to be able to prove that in our patients the damage was not ischemia-related but domoic acid-related and that is probably what will be mentioned tomorrow.

Question from the floor: Those of you who were involved in the post-mortem of these patients: Is there a case where the retinas and optic tract were examined?

T. Perl: I don't know of any. Could we ask why you ask?

Answer from the floor: Well, it is known that in a wide variety of animals certain cellular layers in the retina have a very high concentration of kainic acid receptors and I was wondering if there was any damage in these patients that died.

T. Perl: When you look in animals, can you visually see that or is it simply a microscopic diagnosis?

Answer from the floor: Yes, it has been shown by receptor autoradiography.

Question from the floor: In the history presented by Dr. Teitelbaum this morning, one point was that 5 out of 5

patients who presented with seizures were resistant to Dilantin®. Do you have any information about the rest of the patients regarding this point?

T. Perl: The rest of the patients who had seizures were outside of Montreal. I can think of 2, and we actually do not have detailed information on how they were treated, but both of them died. One of them died within 24 hours of having seizures and probably didn't get an adequate therapeutic trial and for the other, I don't know.

L. Tryphonas: I would like to make 2 comments, one in response to the question of the previous speaker. In experimental animals, retinal lesions of the same type were consistently found both in nonhuman primates and in rodents. My other comment is that if we came to this Symposium to name this syndrome that if we wait one more day until we have all the pathological data, that maybe helpful.

T. Perl: I still don't feel that we are any further along defining the syndrome. I know that there is one other person in the audience who has worked a lot in the Montreal area with this outbreak and may have some comments on the best way to approach the case definition to use it for practical surveillance, and that's Tom Kosatsky. In terms of the neurologic symptoms, I am not sure if other people have comments but this triad that we used was relatively specific and sensitive and actually was quite useful when we had neurological symptoms. Where we did have problems was with the people who had mild neurologic symptoms. One of them which we argued a lot about and we decided probably wasn't necessarily a manifestion of neurologic disease was dizziness. It could have represented hypovolemia or many other things and we found that was so nonspecific that it wasn't useful. The other thing that we commented on was loss of balance and again we found, that in terms of identifying the disease, that wasn't very useful unless it was documented as true ataxia on a neurological exam, that loss of balance meant too many things to too many people. So, in addition to the triad, the other useful information that we were able to gather was these other objective signs and we looked for change in consciousness, seizures, and cranial nerve palsies. I was wondering if people thought that this would be a reasonable part of the definition or if they thought there would be a better way of approaching it or if they would add things.

J. Teitelbaum: I agree that loss of balance and dizziness are things that you absolutely cannot include and everyone has a different definition of both those terms. Every patient will have a different definition. Memory loss is difficult to put in the acute presentation because we did not document that as part of the acute presentation. It could only be proven where the patient was alert enough and cooperative enough to take the testing, that would prove it, so I don't think you can put that into any type of definition of the acute disease. What was typical was confusion and disorientation as these go together — patients who are confused are almost always disoriented and have changes in the level of consciousness. These things are constant and I think they are the most important. These patients would become encephalopathic and maybe the name should really be "shellfish-related encephalopathy," although this is a much more general term. Only when they are alert can you say that they have a memory loss of true amnesia.

Question from the floor: To follow up with regards to the loss of coordination, motor abnormalities: Am I to presume that there were no consistent motor abnormalities in the human patients? Because in the rodents, mice especially, it is very definitive. The motions, especially forward motions and the chewing motions and so on, always occur and they always occur in exactly the same place in the sequence of symptoms. That wasn't manifested in the humans?

J. Teitelbaum: Yes, I was placing that with convulsions because as I presented it, it was convulsions and abnormal movements and that included posturing, chewing, myoclonus, and seizures. It was not consistent in all the patients. Those who had convulsions tended to all show this but not all the patients showed that manifestation. Only 5 out of our 14 did and we really had the sickest ones.

Comment from the floor: Perhaps the difficulty over this syndrome is that there is a sequence of events in the development of the full picture and we observed this in animals. Some animals do not develop the full picture but they will stop at any one point. So this should be taken into account.

T. Perl: I think Jamie Hockin verbalized it quite well, that there is a wide spectrum of disease as you just insinuated and the manifestations that we see are almost 98% of the time gastrointestinal, and in a few that we didn't see, we were not sure they didn't exist. We just didn't get that information and then, as you said, there is a certain subgroup that will have the neurologic manifestations.

Comment from the floor: We did a retrospective illness questionnaire on about 100 people who met the initial case definition and they are already a subset of the total group of cases. So, they would have met the case definition up there, but among that group of people having asked them about 2 months later about their illness we found that approximately 95% claimed to have had vomiting acutely after eating mussels and of that same group about 40% claimed muscular profound weakness and paucity of speech, lack of volubility. It wasn't so much inability to express ideas as they described it but a lack of desire or lack of production of normal speech. They just said less. Among the hospitalized cases, did you see the same thing? Might that be useful perhaps in the case definition of the neurological state especially for people with milder forms of illness as opposed to, say, acute memory loss which we also found in about 25%, lower than the other 2 symptoms.

J. Teitelbaum: The lack of speech, it was hard to define what was going on, and you see that in many of the charts it is written as aphasia, expressive aphasia, which would have been a lack of expression. Some of the patients seem to be mute but they didn't respond to pain any more than they responded to spoken words so it was really hard to define this as a hard fact when you are discussing an objective neurological examination. I will agree with you though that we did note these patients did not feel like speaking, literally there seemed to be a lack of will to speak or initiative to speak. I am not surprised that they would have brought that out 2 months later. If I were to put that in a definition I would put it in as a description of the syndrome but I don't know that you could use that

as a hard criterion. Many patients complained of weakness. It goes with dizziness, I mean generally weak. You will have patients who have protracted nausea and vomiting for any reason and they will tell you "I am just weak all over" and this is the kind of symptom that you listen to in neurology but that you cannot use, unless you have some other corroborating finding. In our patients, when you were told, "Oh, I am diffusely weak," unless we saw atrophy in their muscles, unless we saw objective weakness on examination, loss of reflexes or something on the electromyogram, we wrote it in but we couldn't use it as a hard fact.

Comment from the floor: For what it's worth, frequently they mentioned that weakness began at about the same time as the vomiting, so weakness was, if anything, the first indication of neurologic pathology.

J. Teitelbaum: I personally wouldn't put it in as part of the definition, but would certainly keep it in mind in a case description that the patients do complain of such a thing, whatever it might represent.

T. Perl: As you can see, the clinical manifestations are going to be very difficult to put into a definition. At this point would anyone like to address the laboratory diagnostic aspects, whether the best approach would be possibly trying to identify it in the food which, in the case of restaurants, is frequently difficult to recover by the time the patient becomes ill, versus the utility of the assays which have been now studied in the animal models both the fecal as well as the urine.

F. Iverson: Well, we have certainly used some of the assays that are available for some of the analysis and I think Jim Lawrence mentioned that the standard high performance liquid chromatography assay works very well with feces. We can run that one and it works just as well as using it in toxic mussel extract. He also mentioned, and we found the same, that doing it in urine is very much more difficult. Doing it in anything else than feces and/or urine is very, very difficult and we haven't been able to do it in various other substrates, such as blood. At least at levels below 1 part/million.

T. Perl: We frequently see these patients 24, 48, and sometimes 72 and even more hours after the ingestion. How sensitive and specific would this assay be at that point, assuming that there is going to be a delay in the time that they get to a physician and that he or she thinks of the diagnosis?

F. Iverson: Well, I would think that you could collect the stool samples, certainly that would be the choice. You could certainly use the assays to do them.

C. Pinsky: How about Mental Research Institute scanning? It should show up water-laden cells in the hippocampus particularly the CA3 if there has been domoic acid there in sufficiently toxic amounts.

J. Teitelbaum: This will probably be brought up in more detail tomorrow when we talk about the MRI and PET studies. But the MRI scans were not very helpful in the 4 patients who had them done. One became positive at a later date with increased signal in both hippocampae but that was a year later. It was not abnormal. Approximately 3 months later when it was originally done and in the other patients it was normal despite the patients having definite clinical abnormalities.

T. Perl: So it sounds like the consensus would be that we would like to use the fecal assay for the time being and perhaps if the mussels are recoverable that would be ideal also. I have one other question to address to Jeannie Teitelbaum. Were there, as Dr. Pinsky eluded to, any tests that we used in the clinical setting that you found useful at all in terms of perhaps something at least consistent with the illness?

J. Teitelbaum: Pretty much the answer is no. The renal function tests were abnormal, but this could have been due to a multitude of things and it is nonspecific; computed tomography scans were not helpful; MRI was not helpful, maybe PET scan would be something that could be useful but it is quite a difficult test to do especially on someone who is critically ill and in intensive care. EEG was abnormal but was nonspecific. There was nothing there that would tell you that it was domoic acid poisoning and electromyogram changes — well, that's very nice except as we all know that in critically ill patients they can develop neuropathies just due to critical illness and electrolyte abnormalities. So, I don't think anything there would be specific enough to be of use.

T. Perl: Does anyone else have further comments?

Question from the floor: Was the nausea and vomiting treatable with phenothiazine? Did you try to treat it?

J. Teitelbaum: I had a small group. By the time they showed up that was not the major problem and it disappeared on its own. When it was treated it was treated with Gravol^{R} intravenously but I don't know if that had anything to do with the resolution of the symptom.

T. Perl: The only person who may know is Tom Kosatsky. He did collect some of that information in his follow-up study but I am not sure if that has been analyzed yet.

The final comment that I have on this is that we had arbitrarily set time limits for the onset of illness and although almost 90% of our illness occurred within those time limits, we did probably miss a certain percentage of patients and those may not be realistic. We have a range and we have a mean and a median for the onset of these symptoms but setting a limit may mean that you miss a certain percentage of these symptoms and it may not be something that would be useful in the definition.

Comment from the floor: If I can comment on that point: I would tend to agree simply because based on what we have heard here and what we have seen in various laboratory studies there seems to be a great deal of variability in terms of the oral bioavailability of this compound. In view of the fact there is a strong likelihood that there is something specific about the individuals who got sick with regard to kinetics or whatever, having a strict definition of kinetics may, as you say, increase the chances of missing some of the individuals. I think there is a good possibility that some people will be a lot longer absorbing it and therefore showing the symptoms than other people.

T. Perl: Dr. Hynie, I tend to concur with you that it would be a good idea for a group of people to propose a definition that could be reviewed, but I am not sure if this is the best forum? Do you have any suggestions?

I. Hynie: I don't want to make a suggestion now, but perhaps we can discuss it informally later on and try to come up with some suggestion

tomorrow. As you know, there is a panel tomorrow on the need for short-term research and maybe this is one of the topics which should come on it as a topic.

T. Perl: That is a good idea.

Addendum

After the Symposium the following proposed names and descriptions of the syndrome were received:

1. Proposed by J. Hockin and T. Perl.

   Name of the syndrome: "Amnesic Shellfish Poisoning."

   Description of the syndrome:

   - An acute intoxication due to ingestion of molluscan shellfish containing domoic acid.
   - Gastrointestinal symptoms are also universal with onset within hours to 1 day of one or more of nausea, vomiting, anorexia, abdominal cramps, diarrhea.
   - Some cases complain of persistent severe headache or short-term disturbances of balance or vision.
   - In more severely affected cases, an acute confusional state occurs with onset in hours to 3 days; prominent signs may include any of the following:
     - disorientation, confusion, paucity of speech up to 2 weeks duration;
     - autonomic nervous system dysfunction lasting days to a few weeks (arrhythmias, blood pressure swings, hiccups, excessive bronchial secretion requiring intubation, sweating, low grade fever); and
     - seizures, abnormal ocular movements, grimacing, posturing, myoclonus, loss of reflexes.
   - Death has occurred.
   - Prominent late sequelae appear to be confined to loss of intermediate visuo-spatial recall and mononeuropathies.
   - No specific laboratory findings acutely, although leucocytosis, nonspecific EEG abnormalities and increased creatine phosphokinase have been found; abnormal MRI, PET and electromyogram findings at 2 to 3 months.
   - Domoic acid may be detectable in hot acid extracts of stools in the first few days following consumption.

2. Proposed by C. Pinsky, R. Bose, and G. Glavin.

   Name of the syndrome: "Neurovisceral Toxic Syndrome."

   Description of the syndrome:

   "A syndrome first observed following ingestion of domoic acid-contaminated cultured mussels from waters wherein high concentrations of *Nitzschia pungens* are found. It is characterized by several of the following acute symptoms (in approximate chronological order of appearance): nausea, vomiting, neurogenic gastric distress, gastric bleeding, diarrhea, dizziness, confusion, weakness, lethargy, somnolence, headache, coma, and seizures. There may also be a chronic sign, that of a severe short-term memory deficit, devoid of dementia, which resolves very slightly and very slowly over time."

12. *Excitotoxicity: An Overview*

J.W. Olney

Introduction

A great deal of new information has been generated over the past decade in the excitatory amino acid (EAA) field. Not only have endogenous EAA such as glutamate (Glu) and aspartate (Asp) become recognized as the leading neurotransmitter candidates at the majority of excitatory synapses in the mammalian central nervous system, but it is becoming increasingly evident that the well established neurotoxic (excitotoxic) properties of Glu and Asp or their excitotoxic analogs may play important roles in the pathophysiology of neurodegenerative diseases. Three EAA receptor subtypes have been identified, the kainic acid (KA) and N-methyl-D-aspartate (NMDA) receptors being the best characterized thus far. In the mid-1970s, KA and its receptor system attracted considerable attention. However, over the past decade, the focus has shifted to the NMDA receptor system because of evidence suggesting it may be involved in a wide range of both neurophysiological and pathological processes. Antagonists that powerfully block both the excitatory and neurotoxic actions of EAA agonists at the NMDA receptor or associated ion channel have been identified and are being developed as potential neuroprotective agents for use in the management of neurological disorders. The 1987 mussel poisoning incident linking domoic acid, a KA receptor ligand, to an acute neurodegenerative food poisoning syndrome, promises to generate a new wave of interest in the potential involvement of KA receptors in human neurodegenerative disorders. Evidence suggesting that elderly individuals may be particularly vulnerable to domoic acid neurotoxicity is of interest as it suggests that KA receptors may be sensitive mediators of excitotoxic events in the elderly. In this article, I will review information linking excitotoxic mechanisms to neurodegenerative processes with an emphasis on both NMDA and KA receptors because there is basis for believing that both receptor systems may play important roles in domoic acid neurotoxicity.

Excitotoxicity: historical perspective

Three decades ago, Curtis, Watkins and colleagues[1], using newly developed micro-electrophoretic techniques to examine the membrane-depolarizing properties of Glu and related compounds, characterized the structural requirements for interaction with an apparent EAA receptor. However, the myriad metabolic involvements of Glu, its ability to excite neurons throughout the central nervous system and the lack of any known mechanism for terminating its excitatory action led neuroscientists of the 1960s to reject Glu as a transmitter candidate. This view prevailed for 2 decades before yielding slowly to evidence suggesting that Glu satisfies the criteria for a transmitter. Finally, in the 1980s, Glu has gained widespread acceptance as the front-running transmitter candidate at the majority of excitatory synapses in the mammalian CNS.

Since Glu is found in very high concentration in the CNS and serves a number of important metabolic functions, early reports that systemic administration of Glu to infant mice destroys neurons in the retina[2,3], or in certain regions of brain[4], were met with disbelief. However, CNS damage following either oral or subcutaneous administration was readily reproduced in a number of animal species, including primates[5]. In addition, it was shown[6] that specific Glu analogs (Figure 1) known to share the neuroexcitatory properties of Glu mimic its neurotoxic effects, that these analogs have a parallel order of potencies for their excitatory and toxic actions, and that analogs lacking excitatory activity also lack neurotoxicity. Moreover, ultrastructural studies[3,7] localized the apparent site of toxic action to postsynaptic dendrosomal membranes where Glu excitatory synaptic receptors are located. These and related observations gave rise to the excitotoxic concept that Glu destroys neurons by excessive activation of excitatory receptors on the dendrosomal surfaces of neurons.

Excitotoxin receptors and anti-excitotoxic drugs

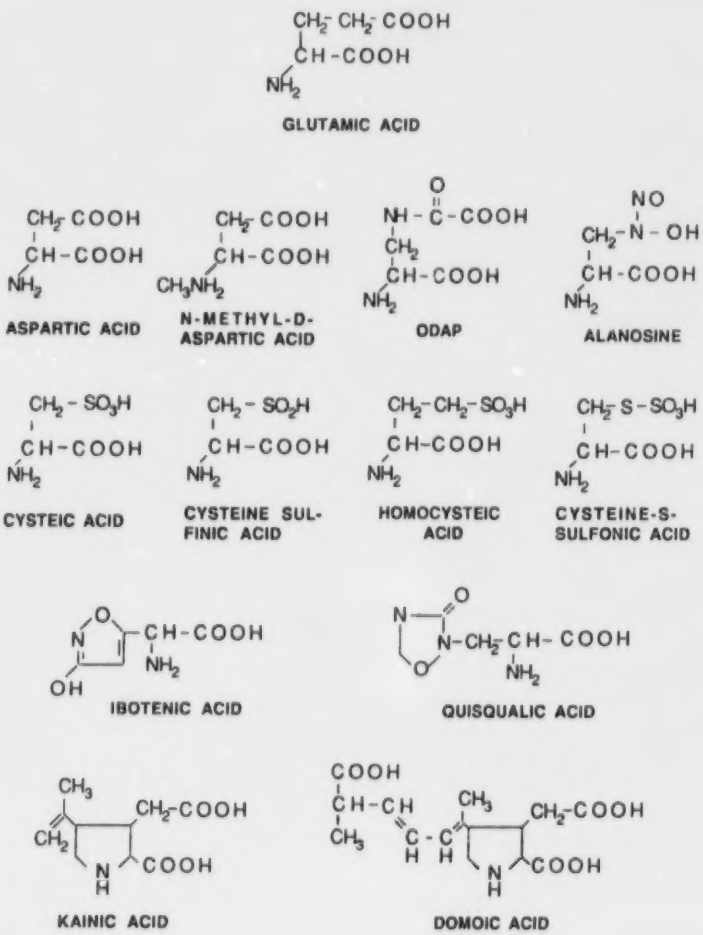
Of major importance for developing better insight into the physiological and pathological properties of EAA transmitter systems was the identification of EAA receptor subtypes differentially sensitive to specific agonists (NMDA, quisqualic acid [Quis], KA) and the discovery of antagonists that block the excitatory actions of EAA agonists at such receptors[8,9]. Shortly after the first EAA antagonists were identified, it was shown that they protect neurons in the *in vivo* mouse hypothalamus against the neurotoxic actions of Glu or its more potent analog, NMDA[10,11]. In further confirmation of the excitotoxic hypothesis, many EAA antagonist candidates have now been systematically screened in *in vitro* preparations and found to have anti-excitotoxic activities corresponding in potency and receptor specificity to their known anti-excitatory activities[12,13,14].

Evidence implicating excitotoxins in neurodegenerative disorders (see below), stimulated interest in the possibility that EAA antagonists might prove valuable as neuroprotective agents in clinical neurology. In Table 1, the results of several EAA agonist/antagonist studies[12,13,15,16] are summarized. The first generation of EAA antagonists identified[9] were competitive NMDA antagonists which compete with NMDA agonists for binding at NMDA receptors. Agents in this class, although possessing moderately potent antagonist properties, are of uncertain value for clinical applications as they do not readily penetrate blood-brain barriers. The most powerful anti-excitotoxic drugs identified thus far are noncompetitive NMDA antagonists which act at phencyclidine (PCP) receptors to block both the excitatory[17,18] and toxic[12,13] actions of NMDA. MK-801, a drug developed by Merck, Sharp and Dohme, is the most potent known compound in this category. Since these compounds do

penetrate blood-brain barriers, they are of interest as potential therapeutic drugs. Certain currently marketed drugs, including dextromethorphan[19] and several anti-Parkinsonian agents[15], are moderately potent noncompetitive NMDA antagonists. Mixed antagonists, such as kynurenic acid and cis-2,3-piperidine dicarboxylate, block the excitotoxic effects of both NMDA and non-NMDA agonists but are of limited interest because of their low potency and inability to penetrate blood-brain barriers. CNQX, a recently described quinoxalinedione[20], has the important distinction of being the first agent found to block the excitatory[20] and excitotoxic[21] actions of non-NMDA agonists more powerfully than it blocks those of NMDA. Certain thiobarbiturates penetrate blood-brain barriers and are moderately potent against both NMDA and non-NMDA agonists.

Figure 1

Excitotoxic Structural Analogs of Glu. Most of the Molecules Depicted here were found Many Years Ago to Mimic Both the Neuroexcitatory and Neurotoxic Properties of Glu. Domoate is an Exception; this Acidic Amino Acid Analog of Glu was originally Isolated from Algae by Takemoto[101] and was Initially Shown to have Potent Neuroexcitatory Activity by Shinozaki[100] but was not Tested Until very Recently for Neurotoxicity. Preliminary Evidence Suggests that it will Probably Prove to be the Most Powerful Excitotoxin that has been Studied to Date. ODAP = the Lathyrus Neurotoxinβ-N-Oxalylamino-L-Alanine, also Abbreviated BOAA.



Acute versus delayed excitotoxic cell death

In vitro ion substitution experiments have provided evidence for more than one mechanism by which excitotoxin-induced neuronal degeneration can occur. In hippocampal cell cultures[22] or in the isolated chick retina[23], neurons degenerate very rapidly when exposed continuously for 30 min to a toxic concentration of Glu or any of its excitotoxic analogs. In either of these preparations, this acute toxic reaction is abolished by the removal of Na+ or Cl- from the incubation medium, but is not affected by the removal of Ca^{++}. However, Choi[24] has described a slow degenerative process triggered by brief (5 min) exposure of cultured neurons to Glu, a process which is facilitated by the presence of Ca^{++} in the incubation medium. Thus, there is basis for believing that excitotoxins can destroy neurons by either an acute fulminating process which is Na+ and Cl- (but not Ca^{++}) dependent, or by a slow process which is Ca^{++} dependent. It is noteworthy that, in the latter case, excitotoxic neuronal degeneration occurs even though the duration of exposure to an abnormal concentration of excitotoxin is only 1/6 as long as in the former case.

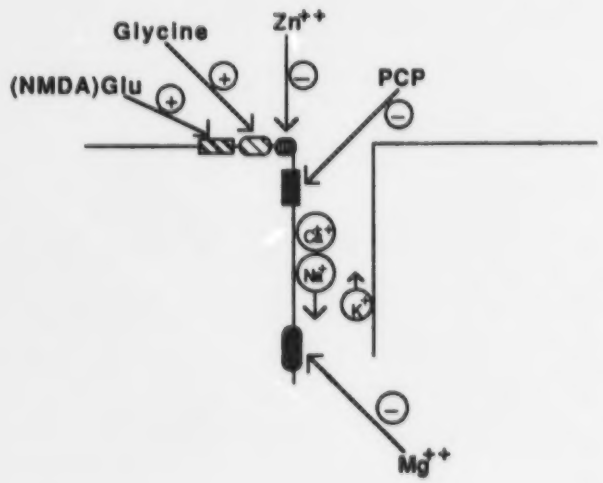
Special features of the NMDA receptor

Of the 3 types of EAA receptor (NMDA, Quis, KA) capable of mediating excitotoxic events, the most studied of these and reportedly the most abundant and widely distributed in the mammalian CNS[25] is the NMDA receptor. Several features of the NMDA receptor distinguish it from other subtypes of EAA receptor. This receptor is linked to a cation channel which has a much higher Ca^{++} conductance than ion channels associated with other EAA receptor subtypes[26], and the NMDA ion channel is subject to a voltage-dependent Mg^{++} blockade[27]. The NMDA receptor is closely associated with a strychnine-insensitive glycine receptor[28] which facilitates opening of the NMDA ion channel and with PCP

Table 1.
Potencies of Antagonists in Blocking NMDA or KA Toxicity
Compounds were rated according to the minimal concentration (μM) required to provide total protection against NMDA (120 μM) or Kainic Acid (25 μM) toxicity. Antagonists were tested over a range of doses from 3000 μM downard. When no blocking was observed at 3000 μM, this is indicated by a dash (-).

Potential Antagonist	vs NMDA (120 μM)	vs KA (25 μM)
Competitive NMDA Antagonists	(μM)	(μM)
D-2-amino-5-phosphonopentanoate (AP5)	25	-
D-2-amino-5-phosphonoheptanoate (AP7)	75	-
D-Alpha-aminoadipate	200	-
Non-Competitive NMDA Antagonists		
MK-801	0.1	-
Phencyclidine	0.5	-
Ketamine	5	-
(±) Cyclazocine	5	-
(+) SKF 10,047	10	-
Dextromethorphan	50	-
Anti-Parkinsonian Agents		
Procyclidine (Kemadrin)	15	-
Ethopropazine (Parsidol)	25	-
Mixed EAA Antagonists		
CNQX	100	15
Kynurenic acid	300	750
(+)-Cis-2, 3-piperidine dicarboxylate	1000	2000
Barbiturates		
Thiamylal	50	250
Thiopental	200	400

Figure 2
A Schematic Depiction of the Various Components Comprising the NMDA Receptor-Ionophore Complex. Recent Evidence Suggests that Endogenous Transmitter (presumably Glu) Released from Presynaptic Axon Terminals Activates NMDA Receptors on Postsynaptic Dendrosomal Membranes Resulting in Opening of a Cation Channel. Glycine, Acting at Strychnine Insensitive Receptors that are Co-localized with NMDA Receptors, Facilitates Opening of this Channel, whereas PCP, Zn^{++} and Mg^{++}, Each Acting at a Separate Site, and Presumably by Separate Mechanisms are Antagonists of Channel Function.



receptors[17,18], which are positioned within this channel permitting PCP agonists to perform an open channel block[29]. In addition, there is evidence that Zn^{++}, acting at a separate site near the mouth of the NMDA ion channel, acts as an inhibitory modulator of channel function[30,31]. Thus, as Figure 2 illustrates, the NMDA receptor system is a remarkably complex entity, the normal function of which depends on a dynamic equilibrium among multiple facilitative and inhibitory factors. It follows that a pathological process affecting any given factor might create an imbalance rendering the entire system malfunctional.

Non-NMDA receptors

In contrast to the abundant information regarding the make-up and physiological properties of NMDA receptors, much less is known about KA receptors and even less about Quis receptors. In fact, much of the electrophysiological evidence, especially from cell culture preparations, has yielded no criteria for distinguishing between KA and Quis receptors, hence the tendency to refer to EAA receptors as belonging to either the NMDA or non-NMDA class. It is possible, however, to demonstrate a differential distribution of NMDA and KA receptors by receptor ligand autoradiographic methods (Figure 3) and, although methods for Quis receptor autoradiography are less well developed, it is reasonably clear that Quis and KA receptors have a different distribution[25,32]. Moreover, it is quite clear that in the isolated chick embryo retina, there are neuronal populations that are sensitive to KA neurotoxicity which are not sensitive to the toxic action of NMDA (Figure 2) or of Quis (not shown). It has been demonstrated, in fact, that the toxic action of KA on a certain sub-population of retinal bipolar neurons is blocked by Quis[33], which makes it unlikely that KA and Quis are simply interchangeable agonist activators of the same receptor complex. One of the most remarkable features of the KA receptor system, as reflected by receptor autoradiogaphic methods, is the concentrated localization of KA receptors in the CA3 region of the hippocampus[32] (Figure 3). Although it remains to be established definitively, it

seems likely that these receptors play an important role in a property of KA that distinguishes it from other excitotoxins – its property of inducing persistent limbic seizure activity (discussed in more detail below).

Excitotoxins and neurodegenerative disorders

Given their presence in the CNS in high concentrations and their intrinsic excitotoxic potential, excitatory amino acids such as Glu and Asp have long been suspected of causing neurodegenerative disorders. Over the past decade, evidence supporting such suspicion has begun to unfold, as has evidence for the companion suspicion that, in many cases, receptors of the NMDA type may be major participants in the neurodegenerative process. Participation of non-NMDA receptors also seems likely, but evidence clearly documenting this has yet to be developed, one reason being that antagonists selective for non-NMDA receptors have not been available. Neurodegenerative conditions in which an excitotoxic mechanism has putatively been implicated include neurolathyrism, sulfite oxidase deficiency, olivopontocerebellar degeneration, amyotrophic lateral sclerosis, hypoglycemia, epilepsy, anoxia/ischemia, CNS trauma, dementia pugilistic, Huntington's disease, Alzheimer's disease and Parkinsonism [reviewed in Olney[34]]. In view of the potential complicity of excitotoxins in these several neurological disorders, considerable interest is currently being focused on the possibility that excitotoxin antagonists (anti-excitotoxins) might be effective in the treatment or prevention of such conditions.

Efficacy of anti-excitotoxins in preventing neurodegenerative conditions

Anti-excitotoxins have not been tested for their ability to prevent neuronal degeneration in human populations; however, in *in vivo* animal studies they have been shown to protect against brain damage associated with status epilepticus[35,36,37,38], hypoglycemia[39], hypoxia/ischemia[40,41,42,43,44,45,99], CNS trauma[46,47], thiamine deficiency[48], and methamphetamine toxicity (a model related to Parkinson's disease)[49]. The most dramatic protection has been shown in status epilepticus, hypoglycemia and hypoxia/ischemia experiments and the agents used in these studies have, in most cases, been the noncompetitive NMDA antagonists MK-801 and/or PCP. As indicated above, agents active as antagonists at KA and Quis receptors have also been identified (e.g., kynurenate and CNQX), but these compounds are not as potent as the most powerful NMDA antagonists (e.g., MK-801) and they do not readily penetrate blood-brain barriers.

The unique excitotoxic properties of kainic acid

Of the various agents that have been studied extensively for excitotoxic activity in the adult mammalian CNS, KA is the most potent[50]. However, it has been difficult to understand KA toxicity solely in terms of a direct excitotoxic action because, when injected directly into the brain , it displays not only the Glu type of direct excitotoxic neuron-necrotizing actively but, unlike Glu, induces sustained seizures and a seizure-linked pattern of disseminated lesions in various brain regions distant from the injection site[51,52,53,35]. Moreover, when administered systemically it also induces sustained seizures and the same seizure-linked pattern of disseminated brain damage. Although most KA studies have focused on rats, Menini et al.[54] have demonstrated in the baboon that intra-amygdaloid injection of KA results in both local and distant brain damage, the distant lesions following a limbic distribution and being associated with limbic epileptiform activity. A similar pattern of damage (cerebral cortex, hippocampus and thalamus) has been described in the autopsied brains of human epileptics[55].

The seizure-brain damage syndrome induced by KA has the following distinctive characteristics: preconvulsive behaviors commence 30 to 45 min after subcutaneous administration of KA (12 mg/kg). These consist of immobile staring, eye blinking, head bobbing and wet dog shakes. At 45 to 60 min, animals begin rearing on hind limbs with forelimbs and head in clonus and conspicuous salivation and drooling. If pushed over by external force, they regain their balance on hind limbs and resume the "praying" posture until the seizure episode is over. If an object is waved before their eyes or a threatening gesture made, they do not respond.

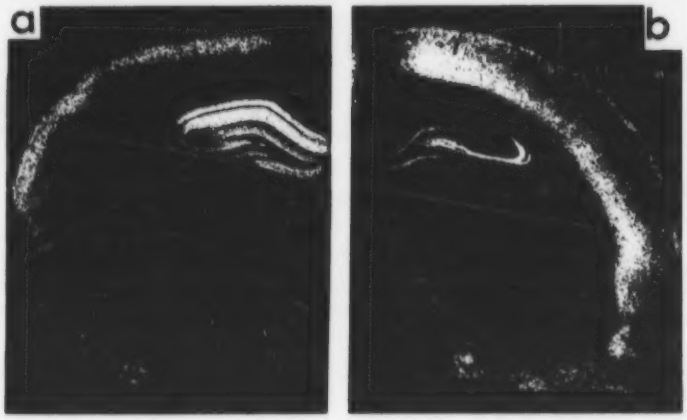


Figure 3
These Autoradiograms were Generated from Rat Brain Sections Incubated in ^{3}H-Glu (panel a) or ^{3}H-KA (panel b). The Recipe Used in (a) Favours Binding to the NMDA Receptor. Thus the Dominant Binding Pattern Seen is that for the NMDA Receptor System which is Particularly Concentrated in the Hippocampus (CA1) and Superficial Layers of the Cerebral Cortex. In Contrast, KA Binding (panel b) is Most Concentrated in the CA3 Region of the Hippocampus and in the Deeper Layers of the Cerebral Cortex.

"Rearing, praying, salivating" episodes recur repetitively every 2 to 5 min for 2 to 6 h. Animals that seize repeatedly at 2 to 5 min intervals are rated as being in limbic status epilepticus. Rats sacrificed for histopathological brain examination after 1 h of status seizures inevitably have a pattern of acute lesions affecting certain limbic and related brain regions involved in excessive discharge activity. The brain regions most characteristically damaged by subcutaneous KA are the piriform and entorhinal cortices, the subiculum and hippocampus, the lateral septum and several amygdaloid and thalamic nuclei. The close relationship between the neuronal discharge activity and brain damage induced by KA has been documented in several studies using depth electrode recordings and 2-deoxyglucose (2DG) autoradiographic methods which show a pattern of electrical and 2DG activation that spreads through limbic and related brain regions in a temporospatial pattern corresponding with the development of acute neurodegenerative changes[35,56,57,58,59].

The role of seizures and endogenous excitotoxins in KA-induced brain damage

In an effort to understand the role of seizures in KA-induced brain damage, we undertook a series of studies aimed at determining whether the same type and pattern of brain damage can be reproduced by other convulsants or other means of inducing persistent seizure activity involving limbic regions of the brain. We found that a wide variety of convulsants (dipeperidinoethane, certain folates, bicuculline, cholinergic agonists or cholinesterase inhibitors in combination with lithium, anti-cholinesterase nerve gases such as soman) induced persistent seizure activity if appropriately applied and inevitably, after approximately 1 h of such activity, early signs of neuronal degeneration could be detected in brain regions involved in the seizure activity[53,60,61,62,63]. Since the type of cytopathology in each case appeared to be the same regardless of the inductory method employed, and in each case it closely resembled the type of cytopathological reaction seen in the arcuate nucleus of the hypothalamus following subcutaneous administration of exogenous Glu[7], we postulated that excessive release and accumulation of endogenous Glu at EAA receptors was responsible for this type of brain damage[64,65]. To further study the potential role of endogenous Glu, we applied a prolonged stimulus (either chemical or electrical) to certain pathways (the corticothalamic and perforant path) which are thought to use Glu as transmitter and which terminate discretely in focal brain regions that can readily be analyzed for neuropathological changes (the thalamus and hippocampus respectively). We found that this consistently produced Glu-like lesions affecting the post synaptic dendritic and somal structures innervated by the stimulated glutamergic pathway[66,67]. Moreover, in the corticothalamic hyperstimulation experiments, we demonstrated that systemically administered NMDA receptor antagonists (MK-801, PCP or ketamine) protect against this type of brain damage[68,38].

Preventing KA-induced brain damage

Ben Ari et al.[52] have demonstrated that the anticonvulsant, diazepam, prevents the seizures and distant but not local pattern of brain damage induced by intra-amygdaloid injection of KA. We have shown that certain anticonvulsants (diazepam and phenobarbital) effectively suppress KA-induced seizure activity and provide substantial protection against brain damage, whereas other anticonvulsants (Dilantin[R], valproate) do not inhibit either the convulsions or brain damage[69]. In this study, we did not actually record seizure activity from depth electrodes and, therefore, cannot be certain to what extent seizure activity was actually arrested in subcortical brain regions. In more recent studies, we have found that various non-competitive NMDA antagonists (MK-801, PCP, ketamine) protect all brain regions except the lateral septum and CA3 hippocampus against seizure-related brain damage induced by KA but do not actually arrest seizure activity in the neuroprotected brain regions (as established by depth electrode recordings)[36,37]. Tentatively, we interpret these findings as follows: Specific anticonvulsants that suppress KA-induced seizure activity protect against KA-induced brain damage because such damage is dependent on persistent seizure activity and seizure-induced release of endogenous Glu. Regarding the ability of NMDA antagonists to protect against seizure-related brain damage induced by KA, we propose that these seizures are triggered by an interaction between KA and its receptors, most likely those concentrated in the CA3 region of the hippocampus (Figure 3), but propagation of the seizure activity entails activation of other circuits and other receptor types, including NMDA receptors. Excessive activation of NMDA receptors (by endogenous Glu) results in destruction of neurons possessing the activated NMDA receptors, and blockade of these receptors prevents these neurons from being destroyed. However, blockade of NMDA receptors does not offset electrographically recordable seizure activity because such activity apparently can be sustained through other transmitter circuits after NMDA receptors are silenced.

Parallels between human mussel poisoning and rat kainate poisoning

In preliminary experiments, we have found that domoic acid is several times more potent than KA in destroying neurons in the chick embryo retina or in causing persistent limbic seizures and seizure-related brain damage in the adult rat. Our preliminary impression is that domoic acid possesses a KA-like neurotoxic profile but may be 3 to 5 times more potent than KA (Stewart, Price and Olney, unpublished). If this proves correct, it will lend credence to the conclusion of the Canadian health authorities that domoic acid was responsible for the poisoning incident in 1987, since the type and pattern of brain damage and other signs and symptoms observed in the Canadian mussel poisoning incident are almost identical to the effects that KA is known to have on adult rats. Even the apparent non-CNS disturbances, such as cardiovascular instability and gastrointestinal distress, can readily be explained in terms of the known effects of KA on the CNS.

Cardiovascular instability: Talman and colleagues[70] have shown that direct application of KA to neurons of the nucleus of the solitary tract (NST) results

in lowering of blood pressure if subtoxic doses are employed, or causes a malignant hypertensive crisis if higher doses are used. Thus, the rapid swings in blood pressure seen in the Canadian poisoning incident may have been caused by an interaction of domoic acid with KA receptors on NST neurons. The NST is a medullary zone immediately adjacent to the area postrema, a brain region that is readily penetrated by blood-borne excitotoxins as it lacks blood-brain barriers.

Gastrointestinal distress: Regarding gastrointestinal symptoms, it is known that Glu is a powerful emetic when administered parenterally to humans[71] and its analogs, including KA, are powerful emetics in dogs and monkeys[5,72,73]; Olney et al., unpublished], and it has been shown that direct injection of KA into the rat CNS causes disturbances in peripheral organs, including necrosis of heart muscle, necrosis of urinary bladder mucosa and gastrointestinal ulcers reminiscent of "stress" ulcers seen in humans following head trauma[74]; Olney et al., unpublished]. Excitatory amino acids cause vomiting by activating neurons in the emesis chemoreceptor trigger zone located in the area postrema to which these agents have free access from the blood.

Convulsions and seizure-related brain damage: The property for which KA is best known is its ability to induce persistent seizures and seizure-related brain damage affecting many limbic and related brain regions, including the hippocampus[35,50,52,64,69,75,76,77,78,79]. Victims of the Canadian poisoning who died and were autopsied were found to have acute lesions in the hippocampus and related limbic brain regions and persistent convulsions were a typical feature of the antemortem clinical picture[80,81].

Mechanism(s) of anterograde amnesia: The NMDA subtype of Glu receptor has recently been linked to memory function in that NMDA antagonists block the induction of long-term potentiation (a putative physiological correlate of memory) both *in vitro* and *in vivo* and have also been shown to impair the performance of adult rats on learning/memory tests[82,83,84,85,86]. The evidence implicating NMDA receptors in long-term potentiation comes largely from studies focusing on CA1 hippocampal pyramidal neurons which are known to have the highest density of NMDA receptors in the brain[25]. Thus, the current thinking is that specific NMDA receptors on CA1 hippocampal neurons may play an important role in memory and that loss of CA1 neurons or dysfunction of their NMDA receptors may result in memory impairment. Conflicting information has been reported regarding the potential role of CA3 hippocampal neurons in memory. Handelmann and Olton[87] described impairment in spatial memory in rats following destruction of CA3 neurons by direct injection of KA into the CA3 hippocampus but Jarrard[88] conducted a similar experiment and did not detect an appreciable impact on memory. It has been shown that ablation of the mediodorsal nucleus of the thalamus (MDT) disrupts memory in the cat[89] and monkey[90,91]. NMDA receptors may be important in the physiology of MDT neurons in that the MDT is a brain region that is typically damaged in the KA seizure brain damage syndrome and MDT neurons can be protected against such damage by pretreatment with an NMDA antagonist[36,37]. I have focused this discussion on CA1, CA 3 and MDT neurons because these 3 neuronal populations have the following in common: 1) all 3 have been implicated in memory functions; 2) all 3 are typically damaged in the seizure brain damage syndrome induced by systemic KA; 3) all 3 were damaged in the brains of victims of the Canadian poisoning incident.

Relationship between excitotoxicity and age

Since there was a strong positive correlation between age and the severity of neuropsychological disturbances among victims of the mussel poisoning incident with a progressively more severe syndrome being seen with advancing age, it is relevant to review what is known about age as a determinant of sensitivity to KA neurotoxicity. It has been shown that KA receptors, although abundantly present in the immature rodent brain, are relatively impotent as mediators of excitotoxicity[92]. Indeed, an amount of KA sufficient to induce widespread damage in the adult rat brain can be injected directly into the infant rat brain with very little neurodegenerative response. The converse is true for NMDA in that nmol amounts of NMDA that would not induce any damage in the adult rat brain can be injected unilaterally into the immature rat brain and it results in neuronal degeneration bilaterally throughout many regions of the developing brain[93,94]. Thus, it is currently believed that NMDA receptors, being relatively hypersensitive in the immature brain, may be exclusively responsible for excitotoxin-induced brain damage in the developing CNS (e.g., in the case of perinatal asphyxia) and that KA receptor's may become increasingly more sensitive and NMDA receptors less sensitive as maturation proceeds. The observation that elderly individuals were especially vulnerable to neuropsychological disturbances following exposure to domoic acid suggests that the KA receptor is as sensitive, and may be even more sensitive, in old age than at any other time in life. Although the NMDA receptor system becomes less sensitive in the age interval from infancy to early adulthood, there is no evidence to suggest that it continues to decrease in sensitivity with advancing age. Indeed, we postulate that domoic acid neurotoxicity is triggered by an interaction between domoic acid and KA receptors but that the cascade of events leading to widespread brain damage may entail the build-up of endogenous Glu at NMDA receptors with consequent excitotoxic degeneration of neurons housing such receptors. If this interpretation is correct, both KA and NMDA receptors may be sensitive mediators of excitotoxicity in old age.

Gastrointestinal absorption of excitotoxins

Since human exposure to domoic acid occurs by oral intake, the question arises whether domoic acid is absorbed freely into the blood from the gastrointestinal tract. While there are no published data on this issue pertaining to any species, the fact that many Canadian consumers developed symptoms of central nervous system toxicity following ingestion of mussels containing high concentrations of domoic acid should be viewed as *prima facie* evidence that the human gut,

at least of some individuals, does absorb domoic acid. The literature pertaining to Glu is instructive on this point; extensive cross-species comparison studies have demonstrated that Glu is absorbed across the gastrointestinal tract much more efficiently in the human than any other species tested to date. For example, a given oral dose of Glu (150 mg/kg) administered orally to adult humans, mice or monkeys caused a 20-fold elevation of blood Glu in humans, a 5-fold elevation in mice and a 0-fold elevation in monkeys[95,96]. It was also observed that there are extreme differences among human adults in the magnitude of the blood Glu elevation produced by a given oral dose of Glu. As I have suggested previously[95,96], these data raise serious questions regarding the advisability of intentionally adding Glu, aspartate or any excitotoxins to the human food supply. The reason for concern regarding Glu and aspartate is that they pass freely across the human gastrointestinal tract and, once in the blood, they pass freely into certain regions of brain that are not protected by blood-brain barriers. This may be particularly dangerous for immature humans because Glu and aspartate interact preferentially with NMDA receptors on neurons in the unprotected brain regions (e.g., the arcuate nucleus of the hypothalamus), and it is a property of immature NMDA receptors to be markedly hypersensitive to Glu or aspartate stimulation[93,94] which renders neurons bearing such receptors hypervulnerable to excitotoxic degeneration . In my opinion, it is unwise to allow unregulated amounts of Glu or any other excitotoxin to contaminate the world food supply (whether the contamination be intentional or unintentional). I consider it particularly unwise to allow total populations to be exposed to an excitotoxin when there is good basis for believing that certain subpopulations may be hypersensitive to the toxic mechanism, for example, young humans may be hypervulnerable to Glu-mediated hypothalamic damage (by an NMDA mechanism), and aged humans to domoic acid-induced limbic degeneration by a KA mechanism.

Prospects for protecting the CNS against neurotoxicity

Since it is not unlikely that domoic acid poisoning will be a continuing threat to seafood consumers and an ongoing challenge for physicians who must provide emergency treatment for such patients, it would be valuable to have an effective antidote for protecting the nervous system against domoic acid poisoning. Although it will be relatively easy by *in vitro* methods to identify agents that can directly antagonize the interaction of domoic acid with its receptor and thereby protect against the excitotoxic effects of domoic acid *in vitro*, it may be difficult to apply the information obtained in such experiments to an *in vivo* situation because most agents currently available that block KA receptors tend to have poor solubility in aqueous solution and do not readily penetrate blood-brain barriers. Therefore, in addition to attempting to develop KA antagonists for neuroprotective purposes, it will be important to explore the neuroprotective potential of certain other classes of drugs.

We have observed in the chick retina that noncompetitive NMDA antagonists (MK-801, PCP, ketamine) do not block KA toxicity even at very high concentrations[12,13], but in the *in vivo* adult rat they do protect many regions of brain against the neurodegenerative changes typically resulting from KA treatment[36,37] and, surprisingly, they confer such protection without substantially reducing the seizure activity in the protected brain areas. Thus, by blocking the participation of NMDA receptors in the electrical storm that KA is known to cause, one can substantially protect against the neurodegenerative consequences of the storm without appreciably quieting the storm itself. Although NMDA antagonists do not confer total protection (e.g., the lateral septum and CA3 hippocampus are not protected), if one can show that they do protect other brain regions (amygdala, piriform and entorhinal cortices, insular cortex, CA 1 hippocampus and several thalamic nuclei) against domoic acid neurotoxicity this would be much better than no neuroprotective therapy at all.

A second principle to be explored is simply the use of anti-convulsants since, to the extent that domoic acid-induced brain damage is seizure mediated, prevention of the seizure activity will prevent the brain damage. Previously we have evaluated the ability of several anticonvulsants to prevent KA-induced brain damage and found that some agents (phenobarbital and diazepam) were relatively effective and others (Dilantin®, valproate) were totally ineffective[69]. Apparently, Canadian physicians who were responsible for the care of the mussel poisoned patients were impressed that various standard anticonvulsants were not effective in arresting the seizure activity. This suggests one of several possibilities: a) domoic acid-induced convulsions may be more difficult to block than KA-induced convulsions; b) it may be more difficult to block this type of convulsion in the human than rat; c) anticonvulsants may block this type of convulsion if administered prior to onset of convulsions but may not be effective if administered after persistent seizure activity is well established. It will be necessary to test various agents for anticonvulsant efficacy in domoic acid treated rats by 2 protocols – administration of the anticonvulsant prior to or at various intervals after domoic acid. The most relevant regimen, of course, would be to administer the anticonvulsant after seizure activity is already evident (since a physician is not likely to see a food-poisoned individual until such symptoms are already beginning to manifest).

One should include thiobarbiturates among the drugs to be tested since these agents are broad spectrum antagonists that block all EAA receptor subtypes[16,97] and they penetrate blood-brain barriers. In fact, they are the only broad spectrum EAA antagonists currently available that do penetrate blood-brain barriers. Also one might test procyclidine, an anti-Parkinsonian drug with anticholinergic activity which we have demonstrated also has NMDA antagonist properties[15]. Recently we found that this agent effectively blocks the seizure brain damage syndrome induced by the powerful cholinergic toxin, soman[98], and is effective even when administered after seizure activity has commenced. Since the anticholinergic properties of procyclidine are stronger than its NMDA antagonist action, we assume that the primary mechanism underlying its protective

action against soman is that it prevents the propagation of seizures through cholinergic pathways. Since it is possible that domoic acid-induced seizures, although triggered by the stimulation of KA receptors, might be propagated through both cholinergic and NMDA pathways, the ability of procyclidine to simultaneously block both cholinergic and NMDA pathways makes it a drug worth testing for its ability to prevent the spread of domoic acid-induced seizures.

In addition to testing single drugs for ability to prevent domoic acid neurotoxicity, one should test combinations (NMDA antagonists, anticonvulsants, thiobarbiturates, procyclidine, KA receptor antagonists) in an effort to increase the neuroprotective efficacy without causing serious side effects. If it is a property of these classes of drugs to have exaggerated and potentially life threatening interactions when administered in combination, it is better to learn this in laboratory rat experiments than in the human emergency room.

Summary

I have reviewed information pertaining to the acidic amino acid analogs of glutamate that have neurotoxic (excitotoxic) activity and pointed out that kainate is the most potent excitotoxin that has been subjected to intensive investigation. Although there is very little published evidence pertaining to domoate neurotoxicity, all available evidence supports the conclusion that the neuroactive properties of domoate are mediated through kainate receptors. Preliminary evidence that domoate powerfully induces a kainate-like seizure-brain damage syndrome in experimental animals further supports involvement of kainate receptors in domoate neurotoxicity. Moreover, the close similarity between this neurotoxic syndrome in experimental animals and the clinical picture witnessed in Canadian victims of mussel poisoning lends further credence to the assumption that this poisoning incident was caused by an interaction between the domoate molecule and kainate receptors in the human central nervous system.

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Discussion:

S. Carpenter, Montreal: You said that MK-801 would protect three areas and I got CA3 and the lateral septal nucleus but I think there is another area.

J. Olney: OK, MK-801 does not protect CA3. It fails to protect CA3 and it fails to protect the lateral septum. It does protect the amygdala and the pyriform cortex and several thalamic nuclei. Those were the major structures that are damaged by kainic acid seizures and the thalamic nuclei and the amygdala and the puriform cortex are all protected by MK-801.

By the way, we put indepth electrodes and measure seizure activity and the seizure activity is continuing in all those regions where MK-801 is protecting it. So, we think that other transmitter systems are maintaining the seizure activity and the damage does not occur because it was being produced by the N-methylaspartate receptor activity.

W. Watson-Wright, Halifax: I was wondering whether any work has been done, or if you know, if there are any kainate receptors in the area postrema?

J. Olney: I think that's an interesting question in relation to the symptoms of domoic acid poisoning. The area postrema is an emesis chemoreceptor trigger zone and glutamate is an emetic compound which produces vomiting; so is aspartate and all excitatory amino acids. We think that they produce vomiting by exciting nerve cells in the area postrema and kainic acid is a powerful emetic compound. I don't know about domoic acid in experimental animals, but I understand it does produce vomiting in humans. So, there are receptors in the area postrema for kainic acid and we think that is the mechanism by which vomiting is induced.

M. Ball, London: We learned yesterday that a specific toxin, a domoic acid in the episode described in the Maritimes was isolated from this diatom creature, that it rose from the phytoplankton growth at certain river estuaries. If we are going to apply any of that to a similar situation for long-term neurodegenerative conditions like Alzheimer's, which is very ubiquitous in populations, one would want to postulate some much more widespread potential food neurotoxin. Is there any evidence from the early literature that you mentioned that domoic acid was ever found in common edible foodstuffs in the orient or for that matter anywhere else in the world?

J. Olney: I think both domoic acid and kainic acid were identified in the folklore of Japan and often in remote regions not necessarily in the general community. They were identified as anthelmintics to destroy intestinal worms and they were ingested in dry seaweed for example, or ground up and ingested, and it was claimed that it did kill intestinal worms. Then they isolated kainic acid and they had a program for a while administering kainic acid to school children – 20,000 school children at a time, to rid the population of the reinfestation with intestinal worms from a reservoir. It is not known whether kainic acid caused any toxic effects in school children because it was being taken with another compound, Santonin, which was another anthelmintic which was known to have some neurological side effects and if there were any neurological side effects of this combination, they ascribed it to Santonin and didn't even think about kainic acid possibly causing it. There is another point that should be made here though and that is, in the rodent, at least, kainic acid isn't very toxic to the central nervous system in infancy and it becomes a powerful toxin to the brain only as the brain matures. So, it is possible that feeding large numbers of school children kainic acid, whatever amount of kainic acid was absorbed across the gastrointestinal tract, it is possible that it wasn't very toxic to the brain just because of the age specificity.

L. Tryphonas: Would you like to comment on the significance of the astrocytic lesions in brains damaged by excitatory amino acids.

J. Olney: The astrocytic lesions – there is astrocytic swelling which we don't view as a primary kind of excitotoxic pathology but rather some kind of reactive response. We aren't sure what the mechanism is of the glial swelling. The other kind of glial response is as we saw in the striatum when kainic acid is introduced: it does cause gliosis. Glial cells hypertrophying and moving into the area but the other kind of glial reaction is a swelling which we think is reversible and I don't think that it has the same mechanism as the neuronal response.

Question from the floor: Yesterday we heard that the patients with domoic acid poisoning initially were given anticonvulsants and these convulsions were very difficult to treat. We do speculate that, nevertheless, some good might have come out of this in view of your demonstration that anticonvulsants prevent the distant but not the local lesions. Secondly, we are going to have a session on therapy later but could you speculate on the possible role of combinations of anticonvulsants, to prevent the distant lesions, and receptor antagonists, to prevent the local lesions, in the therapy of this disorder.

J. Olney: I wonder what anticonvulsants were used. I would think that Diazepam in high intravenous dosage would be probably indicated type of anticonvulsant and I don't know whether that was employed or not. That would be a type of approach that is used for status epilepticus and its possible that the seizures that did occur in humans that ingested domoic acid it is possible that it was subtle enough seizure activity that they weren't thinking of it as status epilepticus and maybe didn't treat it as heroically with anticonvulsants as they would have if they were fully aware that it was persistent status epilepticus. In any event, that's what I would think would be the preferred approach: high intravenous doses of Diazepam. The combined use of an N-methyl aspartate inhibitor with another anticonvulsant might make sense but I would be afraid of possible combined sedative actions of those 2 drug categories and that would have to be tested in experimental animals to be sure that it doesn't depress the central nervous system so severely. For example, Diazepam will cause excessive depression or inhibition through the GABAergic system and then if you also depress the major excitatory system in the brain by antagonizing NMDA receptors you might be overdoing the depression to death. So that should be tested in experimental animals, I think. But if one were to use only the N-methyl aspartate antagonist like MK-801, one would expect that you might protect against brain damage but you wouldn't stop the seizure activity. So, it should be some kind of combination if you want to prevent both of them. I should also mention that we have been studying MK-801 and Phencyclidine quite a bit and we will have a paper coming out in Science in a few months that describes some cerebral corticoneuronal pathology that is induced by these agents. They cause vacuolization of some singulet and retrosplenial corticoneurons which is totally separate from the neuroprotective effects that they have on other neurons. So, that complicates the picture also, for using these compounds as neuroprotective agents; and of course, they also are thought to have psychotomimetic effects, which is if it's just a trench in psychosis, perhaps, one can accept a transient psychosis in trade for protection against permanent brain damage, but it is nevertheless an important consideration.

13. *Neurotoxic Effect of Domoic Acid: Mediation by Kainate Receptor Electrophysiological Studies in the Rat*

G. Debonnel, M. Weiss, and C. de Montigny

Summary

Domoic acid, an excitatory amino acid structurally related to kainic acid, has recently been identified as being responsible for the severe intoxication presented, in 1987, by more than 150 people having eaten mussels grown in Prince Edward Island. The present *in vivo* electrophysiological studies, using unitary extracellular recordings obtained from pyramidal neurons of the CA1 and of the CA3 regions of the rat dorsal hippocampus, were undertaken to study the effect of kainic acid and domoic acid applied by microiontophoresis and compare their potencies to that of agonists of the 2 other subtypes of glutamatergic receptors or neuropeptides. The activation induced by domoic acid and kainate was more than 20-fold more potent in the CA3 than in the CA1 region, whereas no such regional difference could be detected with all the other substances tested. In the CA1 as well as in the CA3 region, domoic acid was about 3 times more potent than kainate. A selective lesion of the mossy fibre system originating from the dentate gyrus and projecting to the CA3 region of the dorsal hippocampus, drastically reduced the excitatory effect of kainic acid and domoic acid in this later area, without affecting the response to the other substances tested. Several class of pharmacological agents were studied in an attempt to find an antagonist of kainate and domoic acid. Only benzodiazepines could selectively suppress the neuronal activation induced by kainate, however, with a lower efficacy in the CA3 than in the CA1 region. These results demonstrate that domoic acid is a potent agonist of kainate receptors and that, in the CA3 region, it might produce its neurotoxic effects through activation of kainate receptors located on mossy fibre terminals. Finally, in the event of a new wave of intoxication, our results suggest that a rapid treatment with high doses of benzodiazepines could possibly prevent the important and irreversible hippocampal damage.

Introduction

Domoic acid has been identified as the substance responsible for the 1987 intoxication of more than 150 people, having eaten mussels grown in Prince Edward Island[51]. Domoic acid, a structural analogue of kainic acid (KA)[6,45], is part of a series of dicarboxylic amino acids which are becoming more and more important as it seems that some of them could be involved in the pathogenesis or in the treatment of several neurological or psychiatric syndromes such as temporal lobe epilepsia, Huntington's disease, Alzheimer's disease, Parkinsonism and schizophrenia[2,3,9,21,27,28,31,32,38].

Some of these dicarboxylic amino acids have been known for several centuries, mostly in Asia where they were used as ascaricids, insecticides and taste enhancers. However, the attention of scientists began to focus on them in 1963, when it was first reported by Curtis and Watkins that glutamate (GLU), which remains the prototype of these dicarboxylic amino acids, induced a neuronal activation when applied locally on spinal neurons [10]. Shortly thereafter, it was reported that GLU could also induce seizures and neuronal degeneration in different areas of the brain when administered locally or systematically[1,8,35]. It soon became evident that these excitatory and neurotoxic properties of GLU were shared by several other molecules and that their excitatory potency was proportional to their neurotoxic effect[8,11,12,25]. It is on this basis that Dr. Olney[37,46] proposed the excitotoxic hypothesis according to which the neuronal necrosis was induced by too high a level of activation. In past years, several studies have been dedicated to the excitatory amino acids. Some results in particular suggest that several of them act as neurotransmitters or neuromodulators in the mammalian brain[39,41,42,43] and that their effects are mediated via 3 subtypes of receptors denominated from the exogenous substances having the highest affinity for each of them, namely KA, quisqualate (QUIS) and N-methyl-D-aspartate (NMDA)[22,48].

The present results concern several series of experiments which were undertaken in order to identify the substratum of the effect of KA and domoic acid. Kainate was a particulary interesting substance as: 1) it was struturaly related to GLU [6]; 2) it was the most potent of the dicarboxylic amino acids, either for its ability to induce an activation or for its ability to induce a neurodegenerescence [5,6,9,29,30,40,44]; and 3) it offered a simple model to verify the excitotoxic hypothesis since it has been well documented that there is a large difference between the CA1 and CA3 regions of the dorsal hippocampus, regarding their sensitivity to the neuronal degeneration it induces[30,34,36].

Material and Methods

Colchicine lesions

Male Sprague-Dawley rats (225 to 300 g) were anesthetized with chloral hydrate (400 mg/kg IP) and mounted in a stereotaxic apparatus. A burr hole was drilled 4 mm anterior to lambda and 2 mm lateral to the midline. The tip of a 5 l Hamilton syringe was lowered into the region of the dentate gyrus, 3.1 ml below the cortical surface. Colchicine (3.5 g or 5 g in 1 L of distilled water), a selective toxin for the granular cells of the dentate gyrus was slowly injected over a 3-min period. The needle was left in place for 1 min in order to avoid a backflow of the liquid into the needle track. During the 48 h following the surgery, animals were injected with a mixed solution consisting of 1 mL of sodium chloride 0.9% and glucose 5% twice daily. The animals were then kept in a dark : light cycle of 12 h : 12 h with food and water ad libitum. Electrophysiological experiments were carried out 3 weeks after the lesion.

Electrophysiological recordings

For the electrophysiological recordings, the animals were

anesthetized with urethane (1.25 g/kg, IP) and mounted in a stereotaxic apparatus. Five-barrelled glass micropipettes prepared in a conventional manner [24] were used for extracellular unitary recording and microiontophoresis. The central barrel, used for unitary recording, was filled with a 2 M NaCl solution saturated with Fast Green FCF. One side barrel, used for automatic current balancing, was filled with a 2 M NaCl solution. Depending upon the experimental series, the 3 remaining barrels were filled with 3 of the following solutions: acetylcholine (ACh; 20 mM in NACl 200 mM, pH 4), chlorediazepoxide (10 mM in NaCl 20 mM, pH 4), cholecystokinin-8-sulphate (10 M in 200 mM NaCl, pH 5), domoic acid (100 M or 1 mM in NaCl 400 mM, pH 8), dynorphin A 1-13 (500 M in NaCl 200 mM, pH 5), flurazepam (50 mM in 50 mM NaCl, pH 4), GLU.HCl (50 mM in 50 mM NaCl, pH 8), ibotenate (IBO; 10 mM in NaCl 200 mM, pH 8), KA (100 M or 1 mM in NaCl 400 mM, pH 8), met-enkephaline (500 M in NaCl 200 mM, pH 4), NMDA (50 mM in NaCl 400 mM pH 8), QUIS (1.5 mM in NaCl 400 mM pH 8).

The electrode was lowered with a hydraulic micromanipulator in the CA1 or the CA3 region of the dorsal hippocampus. Pyramidal neurons of these areas were identified following Kandel and Spencer criteria[26]: long duration (0.8 to 1.5 min) and large amplitude (0.5 to 2 mv) action potentials and presence of complex-spike discharges. The signal amplified and displayed on an oscilloscope, in the usual manner, was fed to a differential amplitude discriminator generating square pulses. These pulses were first fed to a counter from which integrated firing rate histograms were generated and then to an IBM-PC through a Tecmar interface.

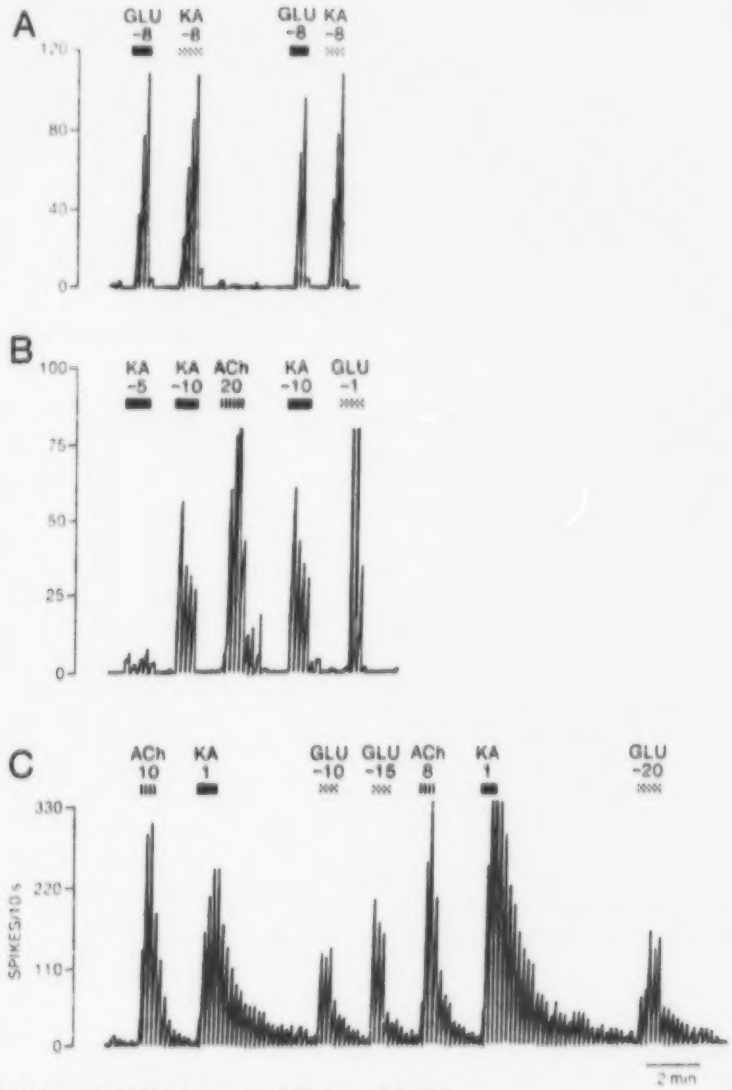
During these 50 s microiontophoretic applications the intensity of the ejecting current for each of the excitatory substances was adjusted in order to obtain a similar and physiological degree of activation (10 to 20 Hz). The time interval between the action potentials and the intensity of the current, as well as the duration of the application, were kept in order to measure the neuronal responsiveness. All excitatory amino acids were ejected as anions, the other substances as cations.

Neuronal responsiveness to the excitatory substances studied was measured by determining the degree of activation; calculated as the number of spikes generated/nA of current used, during a 10 s period or as the total number of spikes generated/nC (1 nC being the charge generated by one nA applied during 1 s).

Histological verifications

At the end of the experiment, a Fast Green deposit was left by passing a -25A current for 20 min through the central barrel. The rat was then sacrificed and the brain removed and frozen at -70°C.

Figure 1
Integrated Firing Rate Histograms Obtained from Unitary Recordings of Parietal Cortical Neurons (A), and of CA1 (B) and CA3 (C) Hippocampal Pyramidal Neurons. Each Vertical Peak Represents the Number of Spikes Generated/10 s.
Bars Indicate the Duration of Applications for which Currents are Given in nA.
Time Base Applies to Both Traces.



KA: kainic acid; GLU: glutamate; ACh: acetylcholine

Subsequently, 40-m thick cryostat sections were prepared and double stained with crezyl violet and Timm's coloration for heavy metals [47]. This double staining procedure allowed an evaluation of the extent of the destruction induced by colchicine and the verification of the localization of the last recording site.

Results and Comments

The first goal was to determine if the differential sensitivity between the CA1 and the CA3 regions of the dorsal hippocampus to the neurotoxic effect of KA could be found for its neuroexcitatory effect.

In the CA1 region of the dorsal hippocampus, ejecting currents between -5 and -15 nA were necessary to induce an activation of pyramidal neurons. These neurons were also activated by similar currents of GLU and acetylcholine (ACh) (Figure 1). In the CA3 region of the hippocampus, neurons were also activated by similar currents of GLU and ACh; however, with KA, a progressive reduction of the retaining current, allowing some KA molecules to leak out from the tip of the pipette, was sufficient to induce an important activation of the neuron (Figure 1). If higher ejecting currents were used, the activation appeared immediately and lasted for a very long period after the end of the application.

In the cerebral cortex, the tested neurons responded similarly to those of the CA1 region (Figure 1).

The results of this experimental series, presented in Table 1, demonstrate that if one takes into account the

Table 1.
Activation of CA3, CA1 and Cortical Neurons by Kainate, Glutamate and Acetylcholine

	Kainate		Glutamate		Acetylcholine	
	Current†	Increase of firing rate	Current	Increase of firing rate	Current	Increase of firing rate
CA3	0.2 ± 0.6	1104 ± 151*	-9 ± 1	1021 ± 176	13 ± 1	755 ± 108
	(43)‡		(43)		(34)	
CA1	-25 ± 4	668 ± 260	-12 ± 2	1522 ± 360	13 ± 0.8	1535 ± 316
	(15)		(15)		(15)	
Cortex	-13 ± 4	1553 ± 605	-12 ± 2	2059 ± 694	13 ± 2	620 ± 157
	(9)		(9)		(9)	

†Iontophoretic currents ± SEM given in nA.
Increases of firing rate for 50 s applications expressed as % ± SEM of spontaneous firing.
‡Numbers of neurons tested.
*P< 0.001, compared with corresponding values of CA1, and of cortex, by analysis of convariance.
SEM = Standard error of the mean.

Table 2.
Ratio Of Potency CA3/CA1

Acetylcholine	0.7
Cholecystokinin-8S	4.8
Dynorphin	1.7
met-Enkephaline	1.9
Glutamate	1.1
Ibotenate	2.5
Quisqualate	1.6
Aspartate	1.8
Kainate	24.1
Domoic Acid	21.3

intensity of the current used, there is a very large difference in the degree of activation induced by KA in the CA3 region when compared to that obtained in the CA1 region or in the cortex. There was, however no significant difference for the other substances (GLU and ACh). Finally, for none of these substances (including KA) was there any difference between CA1 and the cortex [19].

These results suggested that there was a very important difference in the neuronal responsiveness to KA between the CA1 and the CA3 regions.

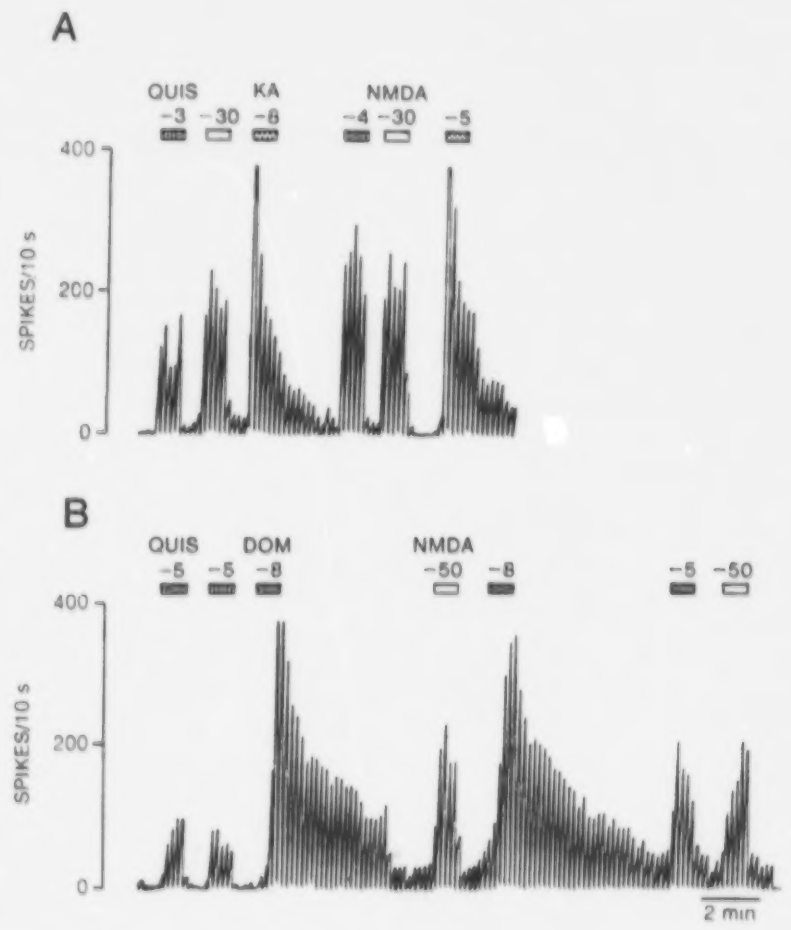
In order to determine if this difference of sensitivity was specific to KA, we used the same paradigm to test several other excitatory substances, either excitatory amino acids or neuropeptides which could have been the endogenous ligand for the KA receptor, given their high concentration in this region.

Due to its structural similarity to KA, domoic acid was one of the substances studied during these comparisons between the CA1 and the CA3 regions.

In the CA1 region of the dorsal hippocampus, due to the very diluted solution of KA and domoic acid (100 M) used in this experimental series, high currents from -80 to -120 nA of KA were necessary to obtain a neuronal response, whereas, ejecting currents of -40 nA of domoic acid induced an activation similar to that obtained with KA. This indicates that domoic acid is more potent than KA.

In the CA3 region, with these very diluted solutions, currents between -5 and -10 nA were required to obtain an activation by KA, whereas similar currents of domoic acid induced a greater activation than that obtained with KA (Figure 2).

Figure 2
Integrated Firing Rate Histograms Obtained from Unitary Recordings Showing the Responses of CA3 Hippocampal Pyramidal Neurons to Microiontophoretic Applications of KA, Domoic acid (DOM), Quisqualate (QUIS) and N-methyl-D-aspartate (NMDA)

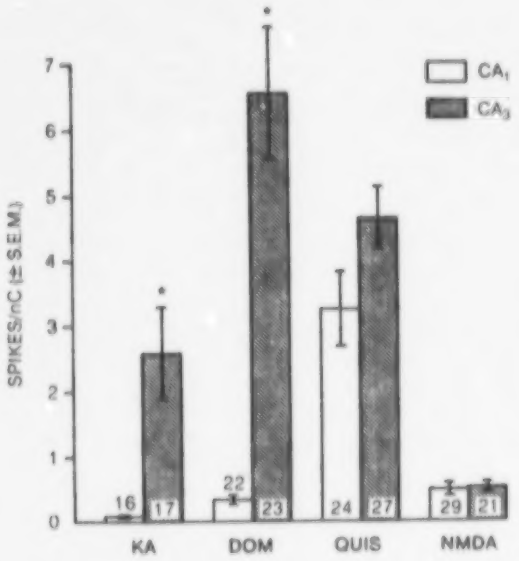


The results obtained in this experimental series are presented in Figure 3. There was no difference between the CA1 and CA3 regions for the 2 control substances (NMDA and QUIS). In this series also, KA was about 20 times more potent in the CA3 region than in the CA1. A similar 20-fold ratio between the CA1 and CA3 regions was found for domoic acid. Moreover, it is noteworthy that either in the CA1 or in the CA3 region, domoic acid was about 3 times more potent than KA (16). This is in keeping with the fact that domoic acid has a 3-to-4 fold higher affinity for [^{3}H]kainate binding sites than KA itself[9,51].

With all the substances tested thus far, when comparing the neuronal response obtained in the CA1 and the CA3 regions, we found that, aside from KA and domoic acid, all of them induced a comparable degree of activation in the CA1 and CA3 regions (Table 2) [14, 15, 16, 18, 20]. These results suggest that domoic acid is acting via KA receptors for which it would be an even more potent agonist than KA.

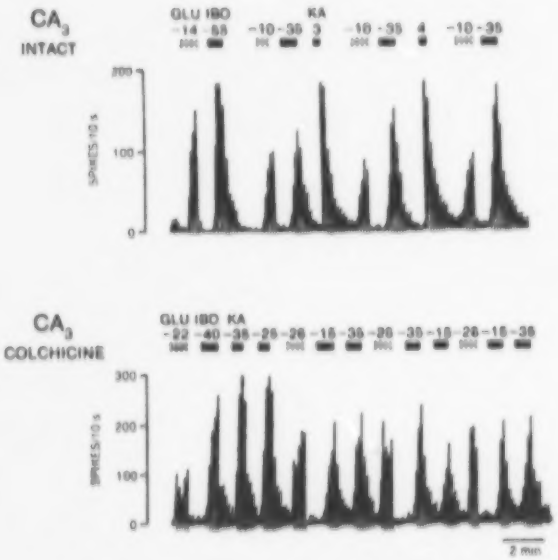
It was then deemed interesting to determine if the effect of KA was postsynaptic (i.e., that KA was acting directly on the pyramidal neurons of the CA3 region) or presynaptic (i.e., mediated by the release of another neurotransmitter). In the latter case, the mossy fibre system originating from the dentate gyrus and giving a selective and massive input to the CA3 region[7] appeared as the most probable substratum of such a presynaptic effect. The same experimental paradigm was used to compare the excitatory response to KA in intact rats and in rats which this mossy fibre system had been destroyed.

Figure 3
Degree of Activation (Measured as Spikes Generated/nC ± SEM) of CA1 and CA3 Hippocampal Pyramidal Neurons by KA, DOM, QUIS and NMDA



• P<0.001 when compared to the corresponding CA1 value (two-tailed Student's test)

Figure 4
Integrated Firing Rate Histograms Obtained from Unitary Recordings of CA3 Hippocampal Pyramidal Neurons Activated by Microiontophoretic Applications of GLU, KA and Ibotenate (IBO) on the Intact Side (A) and on the Colchicine-Lesioned Side (B)



This lesion was obtained unilaterally by injecting, directly in the dentate gyrus region, 3.5 to 5.0 g of colchicine, a selective toxin for the granular cells of the dentate gyrus[23]. About 3 weeks following the injection, there was a virtual disappearance of the dentate gyrus and of mossy fibre terminals on the lesioned side, as revealed by the Timm's coloration counterstained with crezyl violet. However, pyramidal neurons of the CA3 region were not affected.

In the CA1 region, a comparable degree of activation of pyramidal neurons was obtained using similar currents of GLU, ibotenate (IBO), (an agonist of the NMDA receptor)[33], or KA on both the intact and the lesioned sides.

In the CA3 region, on the intact side, a mere reduction of retaining current of KA was sufficient to activate the neurons, whereas currents of GLU and IBO similar to those used in CA1 region were necessary to activate the same neurons. In contrast, on the lesioned side, higher currents of KA (between -25 to -35 nA) were required to achieve a degree of activation similar to that obtained with GLU and IBO (Figure 4).

As presented in Figure 5, the results obtained in this experimental series demonstrate that, in the CA1 region, there was no significant difference between the response obtained on the intact and the lesioned side with all substances tested. However, in the CA3 region, there was a marked (95%) decrease of the response to KA, whereas no significant difference was observed for GLU and IBO[15,20].

In order to confirm that domoic acid was acting via KA receptors, the same experimental paradigm was used. The result of this experimental series demonstrated that, similarly to KA, the lesioning of the dentate gyrus induced a marked decrease of the responsiveness of CA3 pyramidal neurons to domoic acid, whereas the responses to NMDA and QUIS were unchanged (Debonnel, Weiss and de Montigny, unpublished observations).

These 2 series of results first suggest, that domoic acid is producing its neuroexcitatory, and presumably its neurotoxic effect, via the activation of KA receptors and, second that their site of action is presynaptic, as a lesion of the dentate gyrus did produce a drastic

decrease of the potency of KA and domoic acid in the CA3 region. This latter observation demonstrates that the extreme sensitivity of pyramidal neurons of the CA3 region to these substances is only present when the mossy fibre system is intact. The activation of a presynaptic receptor would induce the release of an excitatory neurotransmitter which would in turn activate the pyramidal neurons of the CA3 region. This point is important as it has been demonstrated by the clinical follow-up studies of patients intoxicated by the Prince Edward Island mussels, that these patients presented neurological and neuropsychological symptoms (seizures and memory loss) suggesting an hippocampal lesion. Moreover, in 2 patients deceased following the intoxication, the anatomo-pathological examination has demonstrated a lesion of the hippocampus, much more important in the H3 region than in the H1 region, corresponding to the CA3 and the CA1 areas of the rat, respectively [17], Teitelbaum J, Zatorre R, Carpenter S, et al. [unpublished observations]).

These results suggest that we are dealing with two neurotransmitters which still remain unknown. First, the endogenous ligand for the KA receptor, and second, the neurotransmitter released by the mossy fibre terminals.

The discovery of these 2 endogenous ligands could have important clinical repercussions, but, in the meantime, we attempted to find a selective antagonist for KA, i.e., a substance blocking its neuroexcitatory and neurotoxic effects.

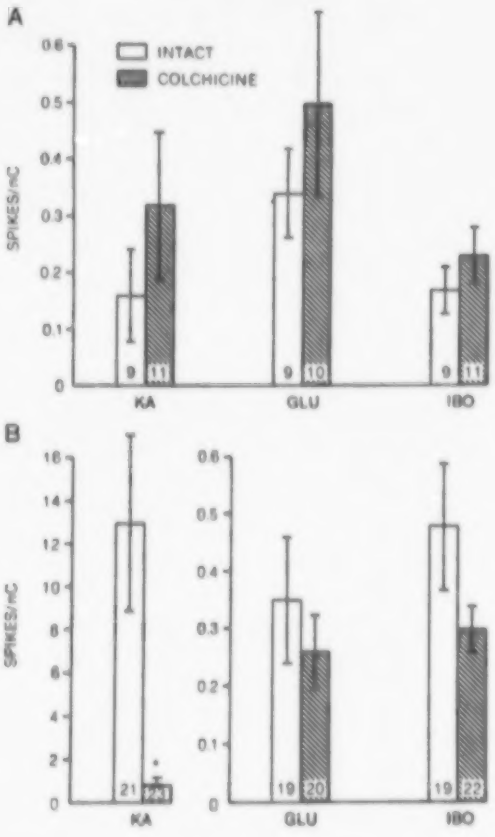
In the CA1 region, pyramidal neurons were activated by KA, GLU and ACh. The intravenous administration of neuroleptics, such as chlorpromazine (4 mg/kg) or haloperidol (2 mg/kg), did not have any effect on KA-induced activation but slightly reduced GLU-induced activation. Phenobarbital administered intravenously at a dose of 10 mg/kg slightly increased the effect of KA. However, the intravenous administration of lorazepam (0.5 mg/kg) (Figure 6) or diazepam (1 mg/kg) produced a near complete suppression of KA-induced activation, whereas it had no effect on GLU- or ACh-induced activations (Figure 7).

Similar results were obtained when flurazepam or chlordiazepoxide were applied microiontophoretically, with low currents (0.2 to 10 nA) directly on the neurons of the CA1 region activated by KA and ACh.

In the CA3 region, however, KA-induced activation was less sensitive to benzodiazepines. Very high currents of flurazepam or chlordiazepoxide (40 to 100 nA) were needed to suppress the excitatory effect of KA. Similar results were obtained with intravenous lorazepam. With this benzodiazepine, a dose of 0.5 mg/kg induced a 90% decrease of the effect of KA in the CA1 region, whereas a cumulative dose of 2 mg/kg produced less than a 50% suppression of the KA-induced effect in the CA3 region (Figure 7) [15].

Another interesting point is that this blockade of KA-induced effect by lorazepam was blocked after an injection of flumazenil (RO-151788), a specific antagonist of the benzodiazepine receptor (Figure 6). This suggests that the effect of benzodiazepines is mediated by the benzodiazepine receptors and is not attributable to a competitive effect on KA receptors, suggesting that benzodiazepines are acting as non-competitive antagonists of KA [18].

Figure 5
Degree of Activation (Measured as Spikes Generated/nC ± SEM) of CA1 (A) and CA3 (B) Hippocampal pyramidal Neurons by KA, GLU and IBO on the Intact and the Colchicine-Lesioned Side



* P<0.001 when compared to the corresponding CA3 value on the intact side (two-tailed Student's test)

Figure 6

Integrated Firing Rate Histograms Obtained from Unitary Recordings of a CA1 Hippocampal Pyramidal Neuron Activated by KA Showing the Response to the Intravenous Administration of Lorazepam and of Flumazenil (RO-15 1788)

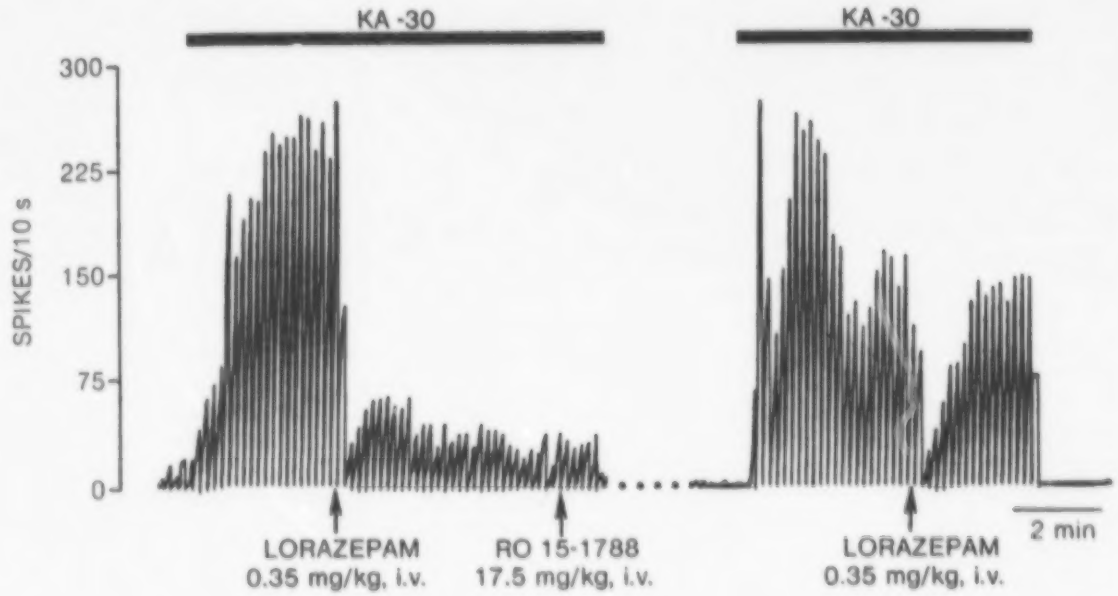
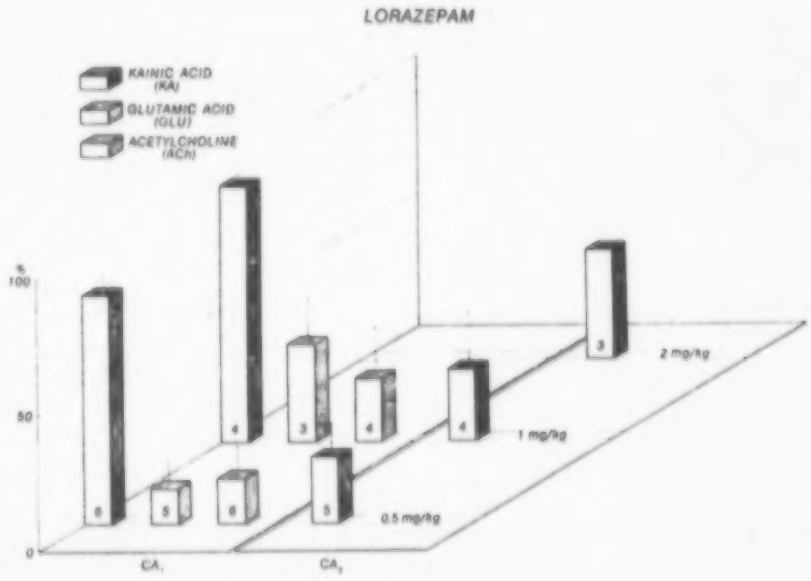


Figure 7

Degree of Suppression* (% ± SEM) Induced by Lorazepam of KA-, GLU- and ACh-Induced Activations of CA1 and CA3 Hippocampal Pyramidal Neurons



* Degree of suppression was measured by comparing the number of spikes generated by each excitatory substance before and 3 min after the intravenous administration of lorazepams

From all the different class of pharmacological agents tested as KA antagonist, only benzodiazepines were able to induce a selective suppression of KA-induced effect, with a less potent effect in CA3 region when compared to the CA1 region.

These results were confirmed by the clinical observation that patients intoxicated with domoic acid and showing seizures, responded to high doses of benzodiazepines (diazepam or lorazepam), whereas phenobarbital and diphenylhydantoin were inefficient.

Conclusions

The results of these electrophysiological studies suggest that domoic acid produces its neurotoxic effect by acting as an extremely potent agonist of KA receptors, and that, in the CA3 region, this effect is mediated by a presynaptic receptor on the mossy fibre terminals. In anticipation of a possible new wave of intoxication by domoic acid, our results suggest that a treatment with high doses of benzodiazepines might prevent the important and irreversible lesions of the hippocampus of these patients. However, it is deemed highly important to undertake further studies to uncover the nature of the 2 neurotransmitters involved in this process. Such a discovery could lead to the synthesis of a potent and selective antagonist for the KA receptor. This antagonist would not only allow a better treatment for patients intoxicated by domoic acid, but could also prevent the irreversible neuronal lesions.

Acknowledgements

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Discussion

J. MacDonald: Teichberg in Israel and Sawada in Japan and ourselves as well, have shown that barbiturates can selectively block the kainic acid channel. I was curious how you can account for that. For example, Sawada showed it in the CA1 region of the hippocampus and it seems to be completely different from your own results.

G. Debonnel: Was it in the same model or was it a neurodegenerative model?

J. MacDonald: In the hippocampus.

G. Debonnel: Yes, but was it electrophysiological model?

J. MacDonald: Yes, the hippocampus. Teichberg in afflux assay and we did it in tissue culture of the hippocampal mass.

G. Debonnel: In tissue culture.

J. MacDonald: Yes. It has basically been isolated cells from brain hippocampal slides similar to your own and cultured cells.

G. Debonnel: Well, I think we had the same comments to some of these results in the Society for Neurological Science and I think that all the presentators have the same result as we have. So, it seems that there is quite a clear difference between the results which could be obtained on slides and the results which are obtained on intact animals at least in this region.

J. MacDonald: So you think the channel has changed *in vivo*. It is not the same anymore?

G. Debonnel: No, I don't think this channel has changed, but I think that some other mechanism could be involved in this process which could change the result.

J. MacDonald: That you may be looking at very indirect effect.

G. Debonnel: That could be. We just know that we put the substance in the surroundings of the neuron. But we never say that we have a direct effect on this target neuron at this level.

J. MacDonald: I am just wondering if you have considered changes in the cell geometry. The CA3 region receives a strong excitatory input, and postsynaptically uses kainic acid receptors. After you have caused a degeneration of the inputs, there may be redistribution of those receptors. In other words, the high concentration of the receptors of the soma may be lost. So, even though you have a high density CA3 at the soma where you were recording postsynaptically with the extracellular electrodes, after the degeneration those receptors are no longer subsynaptic, and therefore like in many other systems they tend to either not maintain that density or they move to other parts of the cell membrane. As a consequence they'd end up looking like CA1 without there having to be a presynaptic receptor.

G. Debonnel: Yes, that could be a solution but at that time we would expect time response relationship and we add at least 4 or 5 experimental series with this kind of lesion with cultures seen from 5 days to about 5 weeks. It seems that after 5 or 6 days when the lesion has really gone down, the effect is completely different between what we have seen in the intact side and the control side. We didn't have the impression that there was a time relationship between the onset of decreasing the response to kainic acid or domoic acid and the time of the injection.

J. Olney: With regard to the question about barbiturates, we have studied the ability of barbiturates to protect against kainic acid toxicity in the chick retina and in that preparation some barbiturates do protect and some don't and it makes a lot of difference what barbiturates you are using. Phenobarbital, for example, isn't very powerful at all in protecting and I think Teichberg found secobarbital was the most powerful agent among the barbiturates that he tested. We found that secobarbital was relatively powerful but we then studied some thiobarbiturates, thiamylal and thiopental and found that they were even more powerful against kainic acid than secobarbital. So, depending on what barbiturate you study, at least in the chick retina, you can either get good protective effects or no effects.

C. Pinsky: Did you test the interaction between any of the antagonists of domoic acid on the neuron activity.

G. Debonnel: Not yet. We are working now very hard on the NMDA receptors but we never tried up to now the interaction between MK-801 and domoic acid. But in regard to the result of MK-801 and kainic acid, I don't expect that we would have much of an effect of MK-801 on domoic acid.

C. Pinsky: Did I understand you to say that you thought it would be desirable to find more specific antagonists in order to consider treatment for any other outbreak of domoic acid poisoning?

G. Debonnel: Yes, I think it would definitely be a good thing to have selective antagonists for each of the 3 types of these receptors, obviously for research reasons, but from a clinical point of view I think that having a very selective antagonist for the kainic or domoic receptors would give us, if proven and at the same time safe, of course, a very much more interesting treatment than benzodiazepam because from rough calculation you would have to use about 200 mg of benzodiazepam for a patient in order to have a good result regarding the seizures induced by domoic acid.

C. Pinsky: But in view of the data which you have shown and your summary there are many receptors, and not only receptors but different regions, presynaptic and postsynaptic, that are affected by the overall syndrome. Might you not consider it to be desirable to have a nonspecific broad-acting antagonist?

G. Debonnel: Well, I think if you have one antagonist for either one of these 2 receptors, you will block the effect anyway. As long as you have one, you are okay. But if you find both antagonists, that would be much more interesting for sure.

C. Pinsky: I just wanted to hear what consideration you might have concerning the domoic acid which, of course, is somewhat different in its action at different receptors from kainic acid, although largely similar.

14. The Molecular Characterization of a Putative Kainic Acid Receptor

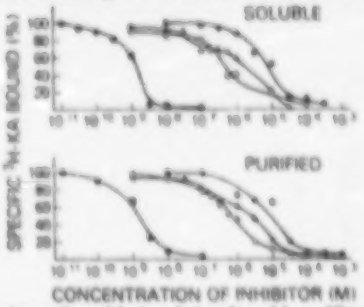
D.R. Hampson, C.J. Dechesne, and R.J. Wenthold

Abstract

The purification of a putative kainic acid receptor has been accomplished by the development of a domoic acid affinity column. Domoic acid, immobilized through the carboxylic acid groups, retains the ability to bind kainic acid receptors in solution. The purified receptor from frog brain migrates as a diffuse band centred at a relative molecular weight of 48,000 on polyacrylamide gels.

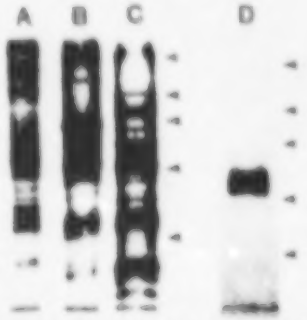
The availability of a purified receptor preparation has allowed the further molecular characterization of a kainic acid receptor through the production of anti-receptor monoclonal and polyclonal antibodies. Monoclonal antibodies that both immunoprecipitate tritium (^{3}H)-kainic acid binding activity and label the molecular weight (M_r) = 48,000 band on Western blots, provide strong evidence that this band is in fact the ligand binding subunit of a kainic acid receptor in frog brain. The use of these antibodies in immunocytochemical studies has demonstrated that the majority of the kainic acid receptor immunoreactivity is concentrated at extrasynaptic sites on neuronal membranes and that a small percentage of the immunoreactivity is located postsynaptically.

Figure 1
Competition Profiles for the Crude Solubilized and Purified KA Receptor Preparations. Binding Assays were Conducted Using 5 nM [^{3}H]KA. Aliquots of the Crude Solubilized or Purified Preparation were Preincubated for 10 min with 6-10 Concentrations of Inhibitor Prior to Performing the Soluble Binding Assay.



■, domoic acid; o, quisqualic acid; ▲, glutamic acid; •, dihydrokainic acid (reproduced from Hampson and Wenthold, 1988 with permission from The Journal of Biological Chemistry)

Figure 2
SDS-Polyacrylamide Gel Electrophoresis of (A) Crude Solubilized Preparation from Frog Brain (10 μg of protein), (B) Pooled Fractions from the DEAE-Sepharose CL-6B Column (2.5 μg of protein), (C) Unretained Fractions from the Purified KA Receptor Fraction (1 μg of protein), (D) Purified KA Receptor Fraction (1μg of protein). Gel Stained with Silver Nitrate.



Markers indicate standards (myosin, M_r = 205,000; phosphorylase b, M_r = 97,400; bovine serum albumin, M_r = 67,000; ovalbumin, M_r = 44,000; and carbonic anhydrase, M_r = 29,000) (reproduced from Hampson and Wenthold, 1988 with permission from The Journal of Biological Chemistry)

Receptors for endogenous excitatory amino acid neurotransmitters, including glutamic, acid and aspartic acid, consist of a heterogenous population of molecules. Based on pharmacological and physiological studies, at least 3 types of receptors have been identified: the N-methyl-D-aspartate (NMDA), quisqualate, and kainic acid receptors (KAR)[1,2]. The neurotoxin, domoic acid, is thought to exert its physiological effects in the central nervous system (CNS) through the KAR[3,4]. A number of studies have indicated that the neuroexcitatory and neurotoxic properties of kainic acid are mediated via KARs.

The molecular characterization of excitatory amino acid receptors has evolved relatively slowly. Several factors, including the absence of methods for the purification of these receptors, have contributed to the slow progress in this area until recently. In 1987 we developed a procedure for the purification of a KAR from frog brain. Frog brain was used as a source of tissue because of the high density of KARs present in the amphibian CNS and because the pharmacological properties of KARs in frog brain (*Rana pipiens*) are similar to those observed in the mammalian CNS[5,6,7].

A key step in the KAR purification procedure is the use of domoic acid affinity chromatography[7]. Domoic acid

covalently coupled to amino sepharose through its carboxylic acid functional groups retains the ability to bind KARs in solution. After extensive washing of the column with a buffer containing a high salt concentration, the receptors are biospecifically eluted with kainic acid. After dialysis, the purified kainic acid receptor displays the same pharmacological profile as the unpurified, solubilized KAR (Figure 1). This procedure provides a highly purified KAR preparation. When subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), the purified receptor migrates as a diffuse band centred at relative M_r = 48,000 (Figure 2). This relative molecular weight is similar to the molecular weight of subunits of several other ion channel receptors such as the glycine[8], gamma-aminobutyric acid[9], and nicotinic acetylcholine receptors[10].

KARs from both frog and rat brain appear to be glycosylated based on their binding to sepharose-conjugated lectins[6,7,11]. We are currently investigating the extent and nature of these oligosaccharide substitutions. Preliminary studies indicate that approximately 10% of the M_r of the KAR in frog brain is composed of carbohydrate. The function of the oligosaccharide side chain remains unknown, although recent studies have shown that the lectin concanavalin A inhibits the desensitization of a related excitatory amino acid receptor, the quisqualate receptor[12].

Further characterization of the KAR is being carried out using anti-KAR monoclonal and polyclonal antibodies. These antibodies were produced by immunizing mice or rabbits with the affinity purified KAR from frog brain[13]. The antibodies are being used in a variety of studies including immunocytochemical localization studies at both the light and ultrastructural level, the immunoaffinity purification of KARs and related molecles, immunoprecipitation experiments, and the screening of complementary (c) DNA expression libraries. Confirmation that the M_r = 48,000 protein purified from frog brain using domoic acid affinity chromatography is in fact a ligand binding subunit of the KAR was provided by the results of immunoprecipitation experiments in conjunction with Western blots. Thus, several anti-KAR monoclonal antibodies that immunoprecipitated solubilized ^{3}H-kainic acid binding activity also labelled a M_r = 48,000 protein on Western blots[13]. Further evidence that the antibodies recognize the major KAR in frog brain was provided by immunocytochemical studies. Tissue sections of frog brain were prepared for immunocytochemical analysis and for ^{3}H-kainic acid receptor autoradiography. The results of these experiments have demonstrated that KARs are widely distributed throughout the frog central nervous system with localized areas showing very high densities of KARs and that the pattern of antibody staining corresonds to the distribution of ^{3}H-kainic acid binding sites[14]. Monoclonal antibodies have also been used to identify KARs in the chicken[15] and pigeon [16] CNS. In both species, the proteins identified have M_r very similar (49,000 to 50,000) to the M_r = 48,000 ligand binding subunit that we have purified from frog brain.

Electron microscopy has revealed a complex distribution of KARs in frog brain at the ultrastructural level[14]. In the optic tectum, the majority of immunostaining is concentrated at nonsynaptic sites on neurons. A small percentage of the labelling was observed at postsynaptic sites. Thus the majority of immunostaining apparent at the light microscopy level is due to staining at extrasynaptic sites. These findings suggest that the same molecule may mediate different physiological functions at the two sites. Future studies will investigate whether or not a similar subcellular distribution of KARs is present in the mammalian CNS.

Although the anti-KAR antibodies raised against the frog brain protein do not appear to recognize native KARs solubilized from rat brain (i.e., immunoprecipitate binding activity), several of the antibodies do label proteins from rat brain on Western blots. One of these monoclonal antibodies labels a M_r = 99,000 protein in rat brain; this band comigrates with the unreduced form of the KAR in frog brain[13]. Localization of this protein at the light microscopy level indicates that it is highly concentrated in several areas of the rat CNS, including hippocampal pyramidal cells and in Purkinje cells in the cerebellum[17]. This protein also appears to be brain specific as it is not present in other tissues of the rat. We are currently attempting to isolate and sequence cDNA clones coding for this protein to determine whether or not the M_r = 99,000 protein in rat brain is homologous with the M_r = 48,000 ligand binding subunit in frog brain [17,18]. We suggest that this rat brain protein may be part of the mammalian KAR or a related receptor subtype.

The molecular characterization of excitatory amino acid receptors has really just begun. In addition to the outstanding questions with regard to KARs noted above, in the larger context of excitatory amino acid receptors there are also several important issues to be resolved. For example, what is the structural and functional relationship between kainic acid, quisqualate, and NMDA receptors? Are glutamate and aspartate the endogenous ligands, or are there additional substances that interact with these receptors? Of critical importance, will be identifying further links between these receptors and neurological disorders. Substantial evidence already exists for the involvement of excitatory amino acid receptors in learning and memory [19] and in several diseases including epilepsy and ischemic brain damage [20,21]. Further characterization of these receptors at the molecular level may help to clarify the role of these molecules in these processes and may also stimulate the development of therapeutically useful drugs.

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15. *The Human Neuropathology of Encephalopathic Mussel Toxin Poisoning*

S. Carpenter

The Montreal Neurological Institute in Montreal, Quebec has had the opportunity of examining the brains of 4 patients who developed neurological symptoms shortly after consuming mussels in November 1987. These patients died at intervals of 7, 12, 24, and 98 days after the ingestion. The death of the last patient, who died almost 3 months after the acute intoxication, cannot be directly related to it, although he was left with a persistent defect in short-term memory and remained hyporeflexic.

These patients ranged in age from 71 to 84 years. They showed various other pathological abnormalities which either preceded or were not directly related to their intoxication. Patient 1 had cardiomegaly and arterial nephrosclerosis, diabetes, cardiac pacemaker, and an old infarct in the right putamen. Patient 2 had extensive amyloidosis in liver, spleen, thyroid, kidney, and intestines. He had a terminal hemorrhagic gastritis and enteritis as well as bronchopneumonia. Patient 3 showed cardiomegaly with moderate coronary artery disease and severe aortic atherosclerosis. He had an old small lacunar infarct in his left amygdala and a recent hemorrhagic infarct in his left parietal occipital lobe. Patient 4 had severe coronary artery disease with cardiomegaly and recent myocardial infarct. His mitral and aortic valves were calcified. He had severe aortic atherosclerosis, pleural and pericardial effusions as well as ascites, severe emphysema, pulmonary edema, and both recent and remote infarcts in his brain.

The lesions ascribable to the intoxication were very similar in their location in the brain in the 4 cases, although the character of the lesions varied with the stage of evolution at the time of death. All showed severe involvement of the hippocampi and subicula. There was moderate sparing in field H2 of the hippocampus. The dentate gyrus was involved in 3 of the 4 cases. All showed involvement of the amygdala and pyriform cortex with general sparing of the lateral nucleus of the amygdala. All showed some involvement of the polymorphic layer of olfactory tubercle, the nucleus accumbens septi, the septal area and the claustrum. Three showed involvement of the insular cortex and subfrontal cortex, limited to the 6th layer in one and also involving the 5th layer in 2 others. The patient without cortical lesions was the one surviving longest. All showed lesions in the thalami, with nucleus submedialis being the area most consistently involved. The cerebellum was not involved, although the second patient had some focal loss of Purkinje cells, which was probably older and unrelated. The only sample of spinal cord was a segment of thoracic cord in case 4, which appeared normal. A sample of peripheral nerve in the same case also appeared normal.

In the patient dying after 7 days, affected areas showed shrunken eosinophilic neurons often with a prominent perineuronal space. There was some tendency for fine vacuolation of the neuropil in severely affected areas of the hippocampus and subiculum. Astrocytic nuclei in affected areas tended to be enlarged and increased in number.

In the brain of the patient dying after 12 days the damaged areas also showed eosinophilic shrunken neurons. Some phagocytosis of neurons had begun, as, for instance, in the most anterior part of the hippocampus, and some neurons appeared to have disappeared completely, as in the end plate of the hippocampus. Enlarged astrocytic nuclei were more prominent, and astrocytes in some areas such as the amygdala occasionally showed cytoplasmic staining. In field H1 of the hippocampus it is of interest that there was no change in the astrocytes. This could indicate severity of local damage as opposed to a later onset. In the stratum oriens of the hippocampus and sometimes in the pyramidal layer there were small pale pink round structures about 10 μ in diameter, which probably represented swollen axonal segments. A few similar structures were seen in this location in the first case also.

In patient 3 dying after 24 days, most of the damaged neurons had disappeared. The main exception to this was in the thalamus, where eosinophilic shrunken neurons were still noted in the medial dorsal and ventral lateral nuclei. In the hippocampus in field H1 there was not only neuronal loss but also collapse of the tissue in the pyramidal layer. The collapsed tissue contained macrophages and some vessels with hyperplastic endothelium. Rare mineralized neurons were seen. Reactive astrocytes were present around the collapsed tissue but seldom within it. Tissue collapse was also seen in the left amygdala in the basal accessory division.

The reactions in the patient dying after 98 days were quite similar to that in the third patient except that no eosinophilic neurons were left. Tissue collapse was seen in field H1 of the hippocampus, whereas other damaged areas merely showed neuronal loss generally with large reactive astrocytes and some residual microglia. Rare mineralized neurons were seen in the collapsed tissue.

There could be a problem in distinguishing the lesions in these cases from those resulting from hypoxidosis. The hippocampus may be affected in both and in a similar distribution. The character of the acute neuronal changes is much the same (at least as far as we can see in post mortem histology), but the small pink axonal swellings noted in the hippocampus in the first 2 cases are not described in hypoxia. The cerebral cortex in hypoxia tends to show laminar necrosis, but this is most often seen in the 3rd cortical layer and seldom is confined to the 6th layer. Predilection sites are watershed areas, not the insulae and subfrontal cortex. The amygdala may be involved in hypoxia, but Purkinje cells and basal ganglia tend to be more susceptible. Lesions in the basal forebrain would not be expected in hypoxia.

A brief comparison can be made between the lesions in these 4 brains and those seen in rats following systemic injections of kainic acid[1]. Lesions were found in the hippocampal formation, specifically subiculum, CA3 and CA1, in the amygdaloid complex and the olfactory cortex overlying it, in the thalamus, and in variable areas of neocortex in layers 5 and 6. Some differences can be noted: for instance, in the human cases there was involvement of the olfactory tubercle and the dentate gyrus.

In summary the lesions in the human brain following severe encephalopathic mussel toxin poisoning are relatively stereotyped with neuronal necrosis, particularly in the hippocampal formation, showing some sparing of field H2, frequently involving the dentate gyrus, also in the amygdaloid complex and overlying cortex with relative sparing of the lateral nucleus, in the claustrum especially anteriorly, in the septal area, frequent involvement of the 6th cortical layer in the insula and subfrontal cortex, discrete involvement of the olfactory tubercle and the nucleus accumbens and variable degrees of involvement of the thalamus. The amnestic syndrome prominent in patients who survived following severe intoxication can be ascribed to the hippocampal damage. Adequate data is not available on peripheral nerves and lower motor neurons.

Acknowledgements

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Discussion

Question from the floor: You mentioned the septal region. Could you comment a little bit more about the damage in the septal area of these subjects?

S. Carpenter: I don't have terribly good sections on it in all the cases, and it is not a region that I am really an expert in. I don't think I can really say much more.

Question from the floor: I am interested in your overall impression actually, regarding neuropathology. Would you consider, in a crunch, that the observations you saw were in fact an exaggeration of the normal aging changes? Secondly, was there depigmentation of the substantia nigra? Thirdly, was there any hypothalamic damage?

S. Carpenter: I could not find any lesions in the hypothalamus. Substantia nigra was virtually normal. You know everybody at that age will have a little bit of pigment in macrophages but it was very slight. Let me see, the second patient did have a few neurofibrillar tangles, rather a few though, and again I would expect those. I don't think in any way these are an accentuation of normal aging changes.

16. *Neuropathology of Experimental Domoic Acid Poisoning in Non-Human Primates and Rats*

L. Tryphonas, J. Truelove, F. Iverson, E.C.D. Todd, and E.A. Nera

Introduction

An outbreak of food poisoning in Canada during the late fall and early winter of 1987 was traced to consumption of cultured blue mussels (*Mytilus edulis* L.) originating in the estuaries of the Cardigan River in Prince Edward Island. Chemical analysis of mussel samples revealed that they were heavily contaminated with domoic acid (DA). Further investigation of this outbreak showed that the occurrence of high levels of DA in these mussels coincided with a heavy bloom of phytoplankton dominated by the pennate diatom *Nitzschia pungens f. multiseries* — a staple of blue mussels that itself contained or was capable of synthesizing large amounts of DA[1,2].

Additional epidemiological findings indicated that there was a highly localized toxicity problem in the Cardigan River and that the toxin was not very potent. It was estimated that affected mussels were consumed by several thousand individuals but only approximately 200 cases of illness were reported. At least 3 fatalities were recorded, mainly among elderly subjects[3,4].

In this presentation we summarize our preliminary pathologic findings in non-human primates and in rats dosed orally and parenterally with toxic mussel extracts or DA found in contaminated mussels.

Methods And Results

Initial approaches

In the early phase of our investigations when we were confronted with an unknown entity, a number of studies were carried out in simians *(M. fascicularis)* and rodents (Sprague-Dawley rats). The purpose of these studies was to define the toxicologic characteristics of the toxic substance present in suspect cultivated mussels harvested from the Cardigan River estuaries in Prince Edward Island. To imitate as closely as possible the conditions of human exposure, toxic mussel extracts and DA were initially administered *per os*. Later, as we gained knowledge, the route of administration was changed from oral to intraperitoneal and intravenous, in order to: a) maximize the use of the minute amounts of DA available at the time, and b) neutralize the effect of emesis and the vagaries of intestinal absorption.

Table 1.
Summary of Experimental Design and Incidence of Clinical Signs in Monkeys (*M. fascicularis*) Given Extract or Crude Domoic Acid *Per Os*

Dose[a] (mg/kg)	Observation Period (days)	Signs GI	Signs CNS
6.62[b]	17	+	-(?)
6.47	20	+	-(?)
5.63[c]	23	+	-(?)
5.89	44	+	-(?)
5.21[d]	22	+	-(?)
0.00[e]	22	-	-
0.00[f]	15	-	-

(?) = Questionable
[a] = Domoic acid equivalent
[b] = MSG was added (at 0.25%)
[c] = DMSO was added (1 g)
[d] = Crude domoic acid in Polybuffer (Pharmacia)
[e] = Polybuffer
[f] = Untreated control

Morphologic studies

Both the monkeys and the rats were killed under deep anesthesia (pentobarbital or chloroform) by exsanguination and intracardial perfusion with heparinized lactated Ringer's solution, followed by 0.2 to 2-3 L of fixative — consisting of 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, or neutral buffered formalin. Sections were cut at approximately 6 and 10 µm thickness and stained with hematoxylin and eosin, (H & E), luxol fast blue, Bodian, and Nissl methods. Selected sections were stained with the periodic acid-Schiff's (PAS) and Holzer's methods. Also, selected semithin sections were stained with toluidine blue.

Total interneuronal vacuolar area in the 5 affected and 5 control rats was estimated morphometrically in the hippocampus using a Zeiss IBAS 2 image analysis system interfaced to a Hamamatsu video camera mounted on a Zeiss Photomicroscope III. Measurements were carried out in CA3a and CA3b fields of the rostral and caudal regions of the hippocampus.

Oral dosing

Monkeys: To investigate the toxic effects of suspect mussel extracts or DA administered orally 2 separate studies were carried out in close succession. The

objective of the first study was to imitate as closely as possible the conditions of human exposure. To this effect, extracts of suspect mussels or crude DA were administered orally to 5 mature cynomolgus monkeys *(M. fascicularis)* (Table 1). In an effort to facilitate DA absorption, 2 of the monkeys were also given either monosodium glutamate (MSG) or dimethylsulfoxide (DMSO). After dosing, the monkeys were observed in their cages for several days (Table 1). Treated monkeys developed clear gastrointestinal disturbances and rather equivocal central nervous system clinical signs. Gastrointestinal disturbances appeared as early as 2 h after dosing and lasted for a maximum of almost 70 h. Among monkeys variation in the time of onset and duration was considerable. The most frequently encountered gastrointestinal disturbances were anorexia, salivation, retching, vomiting, and diarrhea. CNS signs included disorientation, trembling, prostration (fatigue), wet-dog shakes, and withdrawal. The time of onset and duration of the CNS signs varied. These signs appeared as early as 1 h and lasted for as long as almost 100 h. In addition, even in the monkey in which neurologic signs lasted the longest, there was no continuity in the presence of such signs.

The objective of the second study was to establish a dose response relationship. To this effect 4 cynomolgus monkeys *(M. fascicularis)* were dosed with purified DA and observed in their cages for a maximum of 6 days (Table 2).

Nausea and vomiting lasting for periods reflecting dosage levels were observed in 2 treated monkeys. The monkey given 10 mg DA/kg of body weight vomited a considerable amount of the material 1 h and 30 min after dosing. Exact times of onset for the various clinical signs are presented elsewhere in this symposium[5].

At this stage of our investigations histopathologic changes in orally treated monkeys were either minimal, equivocal, or non- existent. The most consistently found change included dark, mostly triangular, neurons surrounded by a clear space. Neurons in the deepest cerebral cortical laminae, especially within sulci, were most commonly affected.

Table 2.
Summary of Experimental Design and Incidence of Clinical Signs in Cynomolgus Monkeys Given Purified Domoic Acid *Per Os*

Dose (mg/kg)	Observation Period (days)	Clinical Signs
10.00	6	+
5.00	4	+
0.50	4	-
0.00*	6	-

*Negative control, no injection

Table 3.
Summary of Experimental Design and Incidence of Clinical Signs and Lesions in Cynomolgus Monkeys (*m. Fascicularis*) Given Purified Domoic Acid Intravenously

Dose (mg/kg)	Observation Period (hours)	Signs		
		GI	CNS	Lesions
0.500	3:42	+	+	+
0.200	4:50	+	+	-
0.050	4:53	+	+	-
0.025	3:55	+	+	-
0.000*	5:00	-	-	-

*Untreated control

Intravenous dosing

Monkeys: Five cynomolgus monkeys *(M. fascicularis)* were dosed once intravenously with purified DA dissolved in physiologic saline (Table 3). Immediately after dosing, the monkeys were placed in their cages and observed continuously for up to 5 h (Table 3).

All treated monkeys developed gagging which appeared 4 to 17 min after dosing and lasted for 4 to 119 min. The duration of signs was directly proportional to the dose level (see Iverson et al. this Symposium).

Typical CNS lesions were only detected in the tractus solitarius of the monkey that was treated with 0.5 mg DA/kg. They consisted of 2 small, bilaterally symmetrical foci of rarefaction containing numerous variably sized vacuoles (Figure 1). Glia reaction was absent.

Intraperitoneal dosing

Monkeys: One monkey was dosed once intraperitoneally with 4 mg of purified DA/kg of body weight. The dose volume was adjusted with physiologic saline to 10 ml.

Within 2 min from dosing the monkey developed incessant empty mastications and salivation, made numerous attempts at swallowing (nausea), and had projectile vomiting. Later it showed weakness, assumed abnormal postures, developed teeth grinding, became lethargic, had a fixed gaze, had repeated retching, and died 3 1/2 h after dosing.

CNS lesions consistent with excitotoxicity were found in the brain and the retina (Figure 2).

Rats: Seventeen female Sprague-Dawley rats were dosed once with 0, 1, 2, 4, or 7.5 mg crude DA/kg of body weight (Table 4). Immediately after dosing the rats were placed in clear plastic bins and observed for a maximum period of 24 h.

Clinical signs, observed in rats treated with 2 mg DA or higher, consisted of scratching of the scapula, varying degrees of seizures and status epilepticus, and crab-like walking[6,7].

CNS lesions were detected only in rats with overt clinical sigs of toxicity and consisted of acute selective

encephalopathy and retinopathy. Details on the regional distribution of CNS damage are reported elsewhere[7]. Briefly, lesions were present in the periventricular organs[8], the limbic system and the hippocampus. Mild selective encephalopathy was characterized by vacuolation of the neuropil, neuronal hyperchromasia and shrinkage with multiple indentations of the somatic profile, and astrocytic swelling (Figure 3). More advanced lesions included neuronal necrosis. Vacuolation of the neuropil was almost twice as extensive in the caudal part than in the rostral end of the hippocampus (Figure 4). Ocular lesions were most often found in the inner retinal layers.

Control rats, as well as rats treated with 1 mg DA/kg body weight, and 3 of 4 rats given 2 mg/kg were unaffected.

Discussion

The results of these investigations show that oral dosing with toxic mussel extracts, or DA (crude or purified) extracted from contaminated mussels, gives rise in the monkey to a transitory toxicity characterized by disturbances of the gastrointestinal tract and the CNS. The rather short-lived toxicity that followed the administration of a substantially high dose of DA (Tables 1 and 2) reinforces the view that DA has considerable difficulty crossing the gastrointestinal-blood barrier[5]. However, from studies carried out to-date, it appears that the quantity of DA that gained entrance into the circulation was sufficient to induce excitation of the CNS.

In contrast to the rather limited response of monkeys treated orally, toxicologic manifestations in the monkeys and rats treated intraperitoneally or intravenously were much more severe and even life-threatening. This finding suggests that, once in the blood stream, DA can easily gain entrance into the nervous system. Periventricular organs possessing an incomplete blood-brain barrier have been reported to be sites through which glutamic acid, a well studied excitotoxin, enters the CNS[9]. It is suggested that DA, a conformational analogue of glutamic acid, uses the same route.

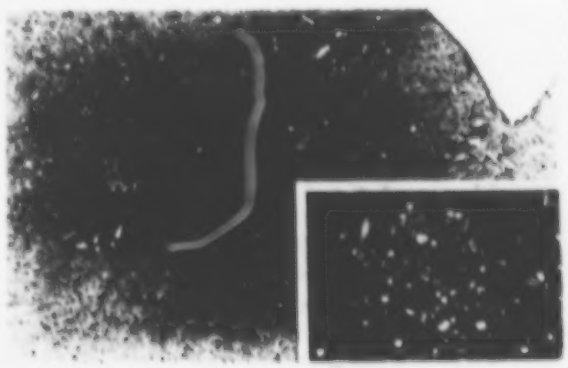
Both the rat and the simian CNS are susceptible to the adverse effects of DA. Both species develop similar lesions which qualitatively resemble those reported for kainic acid[6,8,9]. However, the similarity in the response of the 2 species is not also quantitative. Observed differences concern the distribution pattern of target sites in the CNS (Table 4); and the severity of lesions in a given target. A full explanation for the observed differences in the distribution of target sites is difficult at this time. It has been suggested that sustained epileptiform activity in the brain plays a key role in the development of hippocampal lesions[12,13].

The close resemblance of domoic acid-induced toxicity with that caused by kainic acid suggests that their toxicities share a common pathogenesis. However, additional studies are needed: 1) to determine whether the observed differences in target site distribution and severity of damage between DA and kainic acid toxicity are compound specific or are caused by differences in dosage and length of exposure; 2) to establish whether DA utilizes kainic acid post synaptic receptors or makes use of distinct receptors; and to ascertain the extent of CNS damage produced by extracts of toxic mussels containing DA[7].

The relevance of the experimental findings in explaining the reported toxic mussel brain damage in man is difficult to assess at this point. Our investigations are still in progress and most of our data reflect the results of acute studies carried out in young and young adult healthy animals. Nevertheless, our monkey studies with oral and low intravenous doses reflect to a considerable degree the clinical picture observed in patients who recovered and suggest that the presumed gliotoxic and dendrotoxic CNS damage suffered by such individuals was slight or moderate and therefore reversible[6]. Also, the results show that gastrointestinal disturbances as exemplified by nausea, retching, salivation, and vomiting were not due to a direct irritation of the gastrointestinal tract but rather to a central stimulation of the centre of vomit and are considered to be but one type of excitatory manifestations of DA. The presence of lesions in the area postrema further supports the view that direct local irritation of the center of vomit is responsible for the signs of nausea, retching and emesis. Glutamic acid and its analogues are reported to possess emetic properties[14]. Further studies are needed to determine the threshold for irreversible CNS damage to occur and to understand the role that such underlying factors as a compromised brain blood perfusion and/or accelerated intestinal absorption of DA played in the development of the outbreak of toxic mussel encephalopathy.

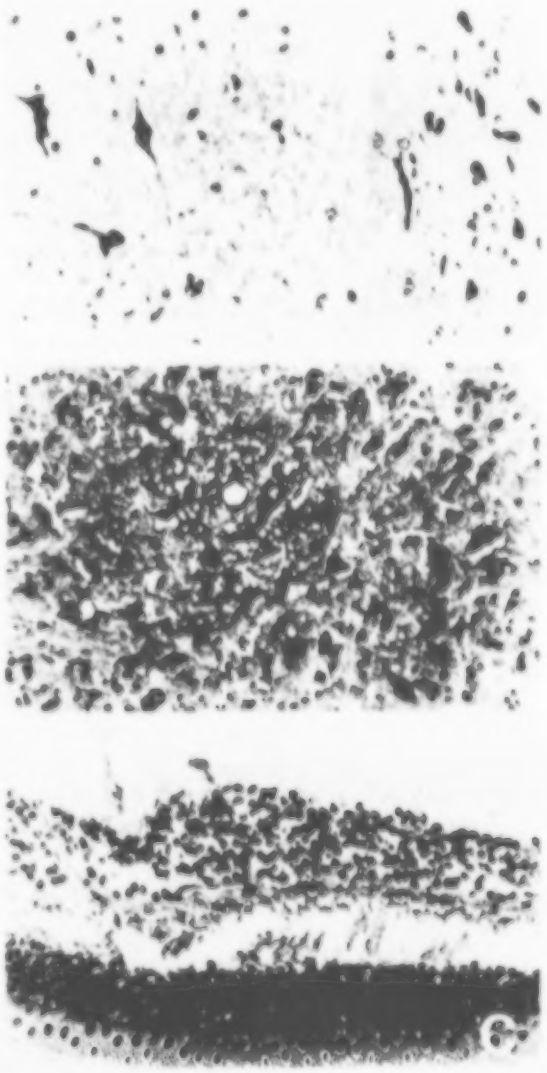
Results reported to date from the human incident and from our toxicity studies indicate that this type of shellfish poisoning is distinct from paralytic shellfish poisoning and neurotoxic shellfish poisoning. Paralytic shellfish

Figure 1
Focus of Vacuolation in the Medulla Oblongata of a Cynomolgus Monkey Treated with 0.500 mg of Domoic Acid/kg of Body Weight IV. H&E. X35. Insert: Same Focus Enlarged. X160.



poisoning is caused by the ingestion of bivalve mollusks contaminated with saxitoxin, a neurotoxin of the dinoflagellates of the genus *Alexandrium* (*tamarense*). Neurotoxic shellfish poisoning is caused by the ingestion of *Gymnodinium breve* contaminated mollusks. Unlike the saxitoxin produced by *Alexandrium* which blocks the propagation of nerve and muscle action potentials and the toxin of *Gymnodinium breve* that causes paresthesias, DA produces a symptomatology and pathologic changes that are characteristic of excitotoxins. It is suggested therefore that the observed human poisoning be called Excitotoxic Amnesic Shellfish Poisoning (EASP). In our view, this term encompasses at least 3 key factors that help define the syndrome: Excitotoxic is suggestive of the mechanism of CNS damage; Amnesic, suggested by J. Hockin and quoted by Wright et al, 1989[15], is indicative of the most serious sequel in affected individuals; and Shellfish points to the food source of the toxin. Also, there seems to be little justification in using the term DA poisoning to define the human intoxication. Patients did not ingest pure DA and at the present time we know little about the human response to pure DA.

Figure 2
Vacuolation in the (A) Area Postrema between N. Hypoglossi (left) and N. Vagus Dorsalis X320, (B) N. Tractus Solitarius X160, and (C)* Inner Nuclear Layer of the Retina X320 of a Cynomolgus Monkey Treated with 4 mg Domoic Acid/kg of Body Weight IP. H&E.



* Photograph C by permission of Toxicologic Pathology, Lawrence, Kansas, USA

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Figure 3
Vacuolation in the (A) Olfactory Bulb X160, (B) Arcuate Nucleus X160, (C) Hippocampus Field CA3 X80, and(D) Inner Nuclear Layer of Retina X320 of a Rat Treated with 4 mg Domoic Acid/kg of Body Weight IP. H&E.

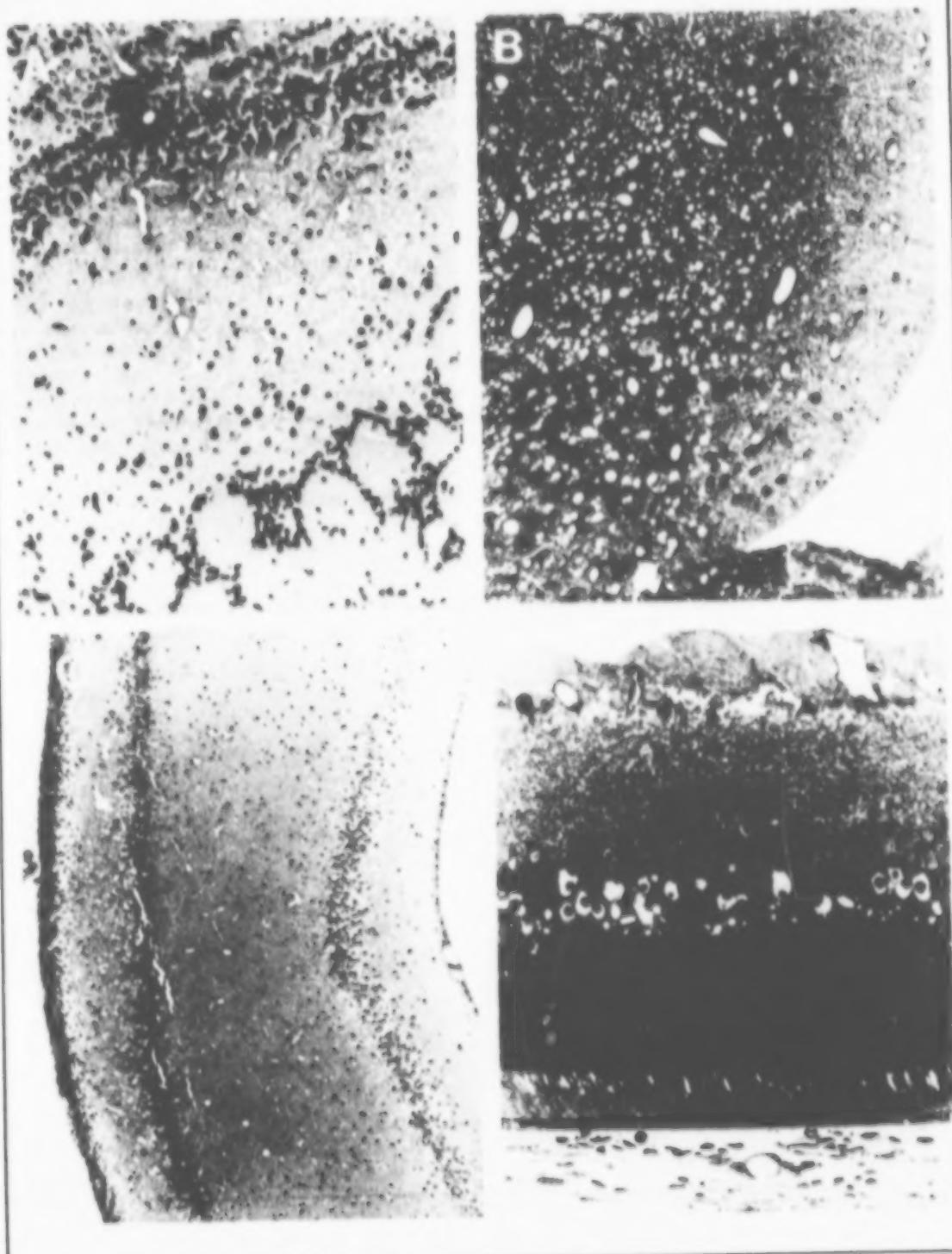

Figure 4
Morphometric Evaluation of Vacuolar Profiles in Field CA3 of the Hippocampus. The Percent Area Fraction of Interneuronal Vacuolation in the Caudal Part of the Hippocampus was Significantly Larger than that Seen in its Rostral End (P≤0.05).

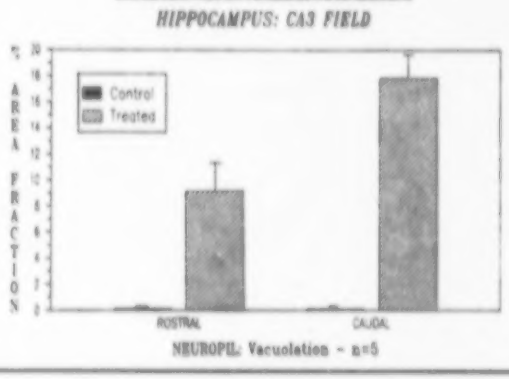

Table 4.
Distribution of CNS Lesions in Monkeys and Rats Treated with Toxic Extracts and Crude Domoic Acid *Per Os*, IP, and IV

Route	Domoic Acid (mg/kg)	Olfactory Cortex	Septal Nucleus	Hippocampus	Hypothalamus	Medulla Oblongata	Retina
Monkeys							
Oral							
Mussel Extr.	5.89- 6.62	-	-	+[d]	-	-	-
Dom. Acid [a]	0.50-10.00	-	-	+[d]	-	-	-
Intraperitoneal							
Dom. Acid [b]	4.00	-	-	+	+	+	+
Intravenous							
Dom. Acid [b]	0.50	-	-	+[e]	-	+	-
Dom. Acid [b]	0.02- 0.20	-	-	-[e]	-	-	-
Rats							
Intraperitoneal							
Dom. Acid [c]	1.00	-	-	-	-	-	-
Dom. Acid [c]	2.00- 7.50	+	+	+	+	-	+

a = Purified or crude D.A.
b = Purified D.A.
c = Crude D.A.
d = Complete pathologic findings were later reported[10].
e = Complete pathologic findings were later reported[11].

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Discussion

J. Olney: I wanted to mention that although we tend to think that subhuman primates are likely to be more similar to man than other species, in studies with oral dosing with glutamate it has been found that gastrointestinal absorption of glutamate is more efficient in the human than in the rodent and much more efficient in the rodent than the monkey. So, if you give a given dose of oral glutamate to monkeys, mice, and man, the species which is least similar to the human is the monkey and it is hard to know how to interpret the findings here with oral dosing of monkeys. It is hard to extrapolate that to the human because sometime monkeys and man aren't alike.

L. Tryphonas: Yes, I fully share that opinion. However, preliminary analytical probing that we carried out in our laboratory shows that the rat does not absorb, or hardly absorbs any domoic acid while it seems that perhaps a non-human primate may be more agreeable to that kind of thing.

17. Excitatory Amino Acids Toxicity in Cerebellar Granule Cells in Primary Culture

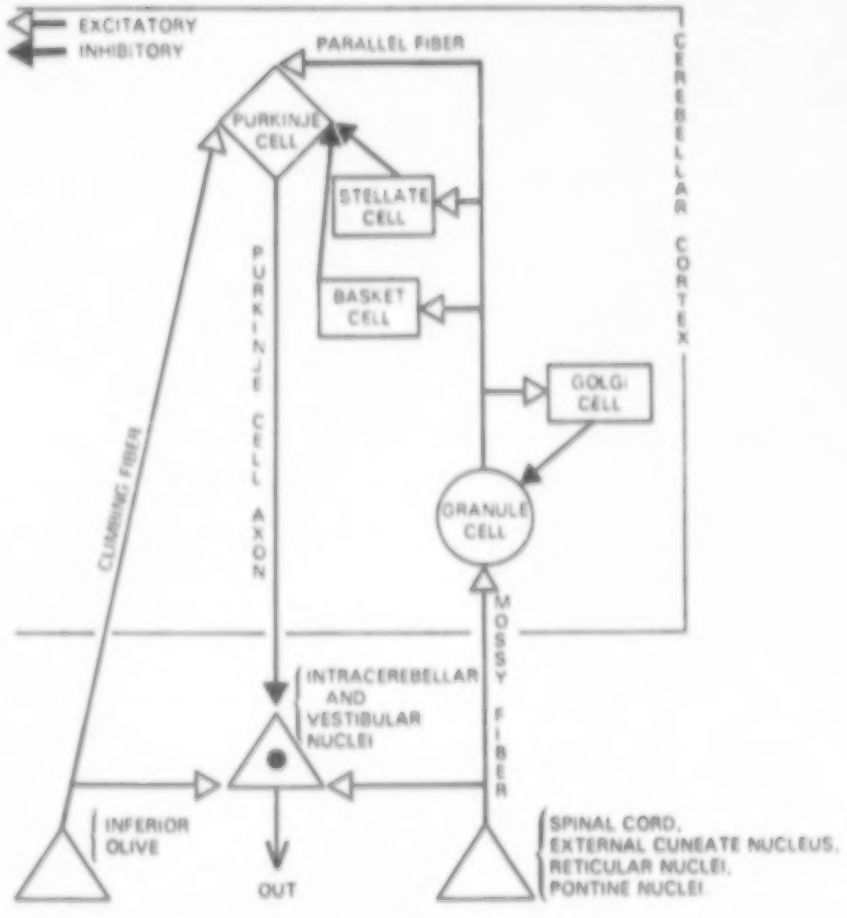
A. Novelli, J. Kispert, A. Reilly, and V. Zitko

Abstract

Primary cultures of cerebellar granule cells represent a valuable neuronal system for the study of the physiology, biochemistry and toxicology of excitatory amino acids[3,6,18,19,20,34]. Using this system, we now report the characterization of the biochemical and toxicological action of a toxic mussels extract, containing the excitatory amino acid domoic acid. The results obtained using toxic mussels extract, non-toxic mussels extract and purified domoic acid, provide evidence for a synergistic action of domoic acid with other potentially neurotoxic excitatory amino acids, normally present in mussels.

In the cortex of the cerebellum there are at least 5 types of neuronal cells: Purkinje, granule, basket, stellate, and Golgi. While Purkinje cells are the only real neurons in the cerebellar cortex because of their inhibitory output, granule cells are the only excitatory interneurons within the cerebellar cortex. Granule cells receive an excitatory input from the mossy fibres[27] and, in turn, they excite the Purkinje cells through the parallel fibres. Therefore, granule cells represent an important station in the elaboration of excitatory inputs and outputs. The excitation is provided through the release of excitatory amino acids (EAAs) endogenously represented by glutamic acid and aspartic acid[9]. The neurotransmitter used for inhibitory purposes in the cerebellar cortex is gamma-aminobutyric acid (GABA)[9]. A scheme of the neuronal architecture in the cerebellum is reported in Figure 1.

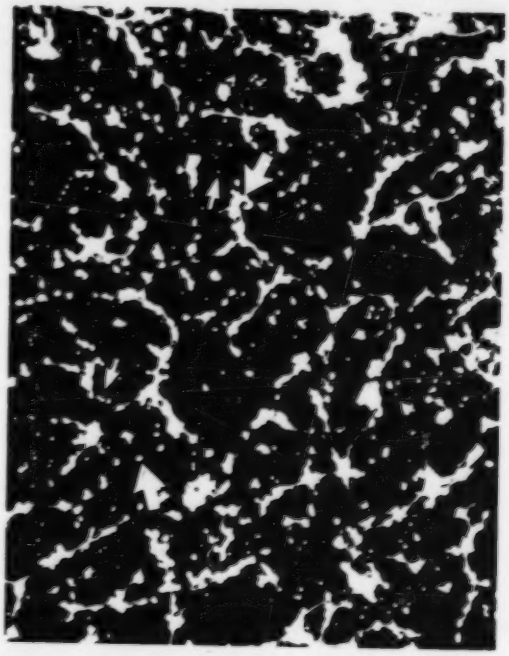
Figure 1
Wiring Diagram of Neuronal Network in the Cerebellum: Granule Cells Integrate Excitatory Input and Output (Details in Text)



In the last 6 to 8 years, rat cerebellar granule cells have been successfully grown in primary culture to a high degree of homogeneity[3,18,20,31]. Granule cells taken from the cerebellum of 8-day old rat pups organize in culture to a final morphology within a few days from seeding, as shown in Figure 2. According to previous studies, where culture were immunostained using antibodies against neuronal proteins, more than 97% of the cell population in culture are neurons[18,31] and astrocytes are present in culture in a limited amount (3%)[18,31]. The majority of the neurons in culture are morphologically identifiable as granule cells, and only 5% of the total neuronal population are expected to be inhibitory interneurons[18], likely stellate. Cerebellar granule cells in primary culture maintain an important feature they possess in vivo: the presence of excitatory amino acid receptors (EAARs)[6,14,18,19,24].

In general, EAARs can be divided into 2 subclasses: N-methyl-D-aspartate or NMDA-type and NON-NMDA-type, according to C.F. Stevens[28]. NMDA-type of EAARs are activated by

Figure 2
Cerebellar Granule Cells† in Primary Culture



†Cerebellar granule cells have been prepared as previously described[20]. After 8 DIC, the culture has been exposed to fluorescein diacetate, a marker for living cells[20]. Under fluorescent light, alive neurons (large arrows) showed a bright green color in the soma and in the neurites. The simultaneous exposure of the culture to ethidium bromide (100 μM x 1 min) resulted in an orange staining of the bare nuclei of dead neurons (small arrows). Similar results are obtained until 30 to 35 DIC. For the experiments reported here, cultures were used between 12 and 21 DIC. Neuronal cultures were supplemented with 5.6 mM glucose every 4 days, and compensated for lost amount of water, due to evaporation.

Table 1.
Antagonism of the Stimulation of cGMP Formation by Excitatory Amino Acids

	% Inhibition of Maximal Stimulation		
	APV	MK-801	PDA
Glutamic acid (100 μM; -Mg^{++})	90	100	0
Kainic acid (100 μM)	10	40	57
Domoic acid (10 μM)	12	12	0

PDA = *cis*-piperidine dicarboxylic acid
APV and PDA were used at 1 mM, MK-801 at 1 μM
Agonists were added for 1 min
Antagonists were added 1 min before the agonists

glutamic acid analogs such as N-methyl-D-aspartic acid, aspartic acid, and quinolinic acid[12]. The channel associated with this receptor-type is gated by Mg^{++} in a voltage-dependent manner[11,21], and allows the passage of large amounts of Ca^{++}[16]. Two types of antagonists are available for the NMDA receptor: 1) the receptor antagonist D-(-)2-amino-5-phos- phonovaleric acid (APV)[12], and 2) the NMDA-channel blocker MK-801[32]. The NON-NMDA type of EAAR is activated by glutamic acid analogs such as kainic acid, domoic acid, and quisqualic acid[1,12]. The channel associated with this receptor-type is not gated by Mg^{++} and allows the entrance of smaller amounts of Ca^{++}[3,10,12,16,30]. The activation of NON-NMDA receptors by quisqualic acid may result in the activation of a G protein which, in turn, activates inositol phospholipid turnover (PI)[29]. No established, specific antagonists for the NON-NMDA receptor are available[12,33].

In cerebellar granule cells in primary culture, EAARs are coupled with guanylate cyclase activity and the consequent production of cyclic guanosine monophosphate (cGMP)[14,19]. Intracellular concentration of cGMP is elevated approximately 50 to 100 fold over basal levels within 1 min following the stimulation by EAARs. The stimulation of cGMP formation by EAAs is dependent upon extracellular Ca^{++} and is common to both types of EAARs, NMDA and NON-NMDA, with different modalities, according to the properties of the 2 types of EAARs. In fact, the stimulation of cGMP formation by NMDA-receptor agonists, such as glutamic acid, is blocked by: 1) extracellular Mg^{++}[19]; 2) APV[19] and 3) MK-801 (Table 1). The stimulation of cGMP formation by NON-NMDA receptor agonists, such as kainic acid, is not reduced by the presence of extracellular Mg^{++}(1 mM)[19], and is weakly antagonized by APV[19] or MK-801 (Table 1). Similarly to kainic acid, domoic acid stimulation of cGMP formation was not potentiated by the absence of extracellular Mg^{++}, and was not inhibited by APV or MK-801 (Table 1). Domoic acid (EC_{50}=3-5 μM) appeared to be approximately 10 times more potent than kainic acid[19], and increased cGMP levels approximately 100-fold over basal levels. Quisqualic

Table 2.
Extracellular Mg^{++} Dependency in the Neurotoxicity by Excitatory Amino Acids Promoted by Low Cellular Energy Levels

Minimal Neurotoxic Concentration (μM)(30 min exposure)

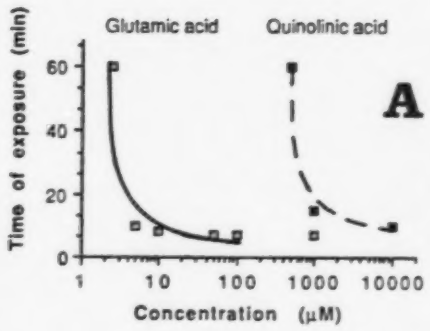
	$-Mg^{++}$	$+Mg^{++}$
Glutamic acid	2.5	25
Quinolinic acid	500	5000
Domoic acid	1.5	2
Kainic acid	20	20

Neurotoxicity was evaluated as described in Figure 3.

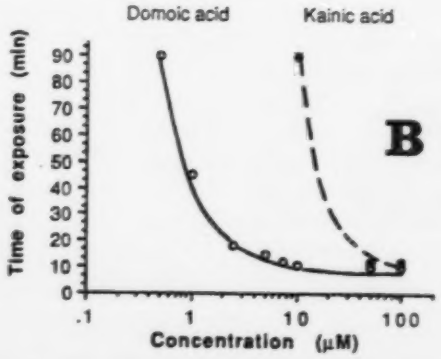
Glucose was omitted from the incubation buffer 30 min. before the addition of the drugs [20]. When indicated, Mg^{++} (1 mM) was omitted from the incubation buffer from the beginning of the experiment.

Figure 3
Timing of Early Signs of Neurotoxicity by Excitatory Amino Acids in Energy-Deprived Cultures

Panel A: Timing of concentration-dependent neurotoxicity by glutamic acid (solid line) and quinolinic acid (dotted line). Neurotoxicity has been induced in the absence of Mg^{++} and glucose from the incubation buffer[20]. Early signs of neurotoxicity have been evaluated timing the occurrence of swelling and loss of refractiveness described in a previous publication[20]. Signs of neurotoxicity occurred approximately at the same time in all the neurons in culture.



Panel B: Timing of concentration-dependent neurotoxicity by domoic acid (solid line) and kainic acid (dotted line). Neurotoxicity has been induced in the absence of glucose and in the presence of Mg^{++} in the incubation buffer[20]. Quinolinic acid and kainic acid were weaker agonists than glutamic acid and domoic acid, and are reported for comparison.



acid, which does not stimulate cGMP[7,19], but rather antagonizes kainic acid action[7], was a potent inhibitor of domoic acid-mediated cGMP increase (Novelli et al., in press).

EAAs are potent neurotoxins. Glutamic acid is considered a potential endogenous neurotoxin in the brain[2,13,17,22,34]. EAAs, such as glutamic acid and aspartic acid, can be ingested within components of a normal diet and may represent a source of potential neurotoxicity[23,26]. Other EAAs, such as domoic acid, may accidentally contaminate some food, and may induce neurotoxicity and death[8,35]. Neurotoxicity by EAAs may be occurring through the 2 types of receptors already described.

In cerebellar granule cells in primary culture, used as an experimental system for the evaluation of neurotoxicity by EAAs, neurotoxicity by receptor agonists, such as glutamic acid and quinolinic acid, occurs via the NMDA-type of EAAR[20]. Neurotoxicity by millimolar concentrations of these EAAs, in the experimental buffer described[20], is limited by physiological concentration of Mg^{++}(1 mM) and by normal adenosine triphosphate (ATP) levels[20]. However, a reduction in the availability of glucose and the consequent decrease of the energy charge (E.C.), defined as the ratio between triphosphate, diphosphate, and monophosphate adenine nucleotides[20], allows the occurrence of neurotoxicity by micromolar concentrations of NMDA-receptor agonists[20]. The factor promoting the toxicity at the NMDA-type of EAAR appears to be a decrease in the voltage-dependent Mg^{++} block of the channel associated with this receptor after reduction of the energy levels. A similar effect is produced using blockers of oxidative phosphorylation, such as potassium cyanide (KCN), or blockers of the sodium-potassium pump (Na+/K+pump), such as oubain, or incubation of the cell culture under anoxic conditions (-0_2). The neurotoxicity induced in all these experimental conditions can be reversed by antagonists of the NMDA-type of EAAR, such as APV or MK-801[20]. As expectable, neutrotoxicity by NMDA agonists is potentiated by the removal of Mg^{++} from the incubation buffer (Table 2). The simultaneous removal of glucose

and extracellular Mg^{++} allows a time-dependent expression of neurotoxicity by NMDA-receptor agonists, at concentrations as low as those stimulating cGMP formation 25 (μM) (Figure 3, Panel A).

We induced neurotoxicity through the non-NMDA type of EAAR by using exogenous EAAs, such as kainic acid and domoic acid. Neurotoxicity by micromolar concentrations of these compounds was rapid in the absence of glucose (Figure 3, Panel B), and, unlike the one induced by NMDA receptor agonists, was not potentiated by the removal of Mg^{++} from the experimental buffer (Table 2). Domoic acid was approximately 10 times more neurotoxic than kainic acid (Figure 3, Panel B), and produced neurotoxic effects at concentrations as low as those stimulating cGMP formation (1 μM).

In the absence of glucose, neurotoxicity by EAAs was dose- and time-dependent but affected irreversibly all the neurons in culture. Consequently, it was difficult to quantify neuronal survival. This result was achieved by long exposure (24 h) to EAAs in the growth medium. Cerebellar granule cells can survive in primary culture for more than a month and EAAs toxicity can be evaluated starting from a steady-state of neuronal functions (Novelli et al., in press). Glutamic acid and domoic acid were approximately equipotent for neurotoxicity (glutamic acid EC^{50} = 11 μM; domoic acid EC^{50} = 7.5 μM). Nevertheless, glutamic acid neurotoxicity was antagonized by APV and MK-801, but not by quisqualic acid, while domoic acid toxicity was only partially antagonized by APV and MK-801 and was completely antagonized only by quisqualate, which has been reported to antagonize neurotoxicity induced by kainic acid, as well[15].

In these experimental conditions, the domoic acid-containing extract from "toxic mussels" resulted to be approximately 10 times more neurotoxic than domoic acid alone. Quite unexpectedly, neurotoxicity appeared to be due to more than domoic acid. In fact the toxicity of the extract was completely antagonized only by the simultaneous presence of APV and quisqualate. This result can be explained by hypothesizing the presence of at least one other EAA[4,25,26], acting as a neurotoxin at the NMDA-type of EAAR, in addition to the already known domoic acid acting at the NON-NMDA-type of EAAR. This hypothesis received experimental support from the finding that extract from non-toxic mussels was indeed neurotoxic to granule cells in primary culture, and the toxicity was completely antagonized by NMDA-receptor antagonists. The possibility that NMDA-receptor agonists, normally present in mussels extract[4,25,26], and domoic acid may synergize in their action was evaluated by measuring cGMP formation as a biochemical index for EAARs activation. The non-toxic mussels extract was slightly effective (approximately 4-fold/basal) in elevating cGMP levels in cerebellar granule cells in primary culture when Mg^{++} (1 mM) was present in the incubation buffer. However, the same extract promoted a large increase (approximately 70-fold/basal) in cGMP intracellular levels when Mg^{++} was removed. Domoic acid at low concentrations (2.5 μM) produced a small (approximately 10-fold/basal) but significant stimulation of cGMP formation in the presence of Mg^{++}. The addition of domoic acid (2.5 μM) to the non-toxic mussel extract, in the presence of Mg^{++}, induced cGMP formation to the same extent as the removal of Mg^{++}. These results indicate that small amounts of domoic acid may potentiate the action of NMDA-receptor agonists, endogenously present in the mussels, by reducng the Mg^{++} block at the NMDA-receptor-associated channel. We are still investigating the neurotoxic effects of the combined action of small amounts of domoic acid and NMDA-receptor agonists present in non-toxic mussels. Based upon our preliminary observation and on the results reported here, we propose a generalized model for the neurotoxic role of domoic acid in mussels (Figure 4). We suggest that domoic acid may simultaneously affect presynaptic and postsynaptic neuronal terminals. At the presynaptic terminals, domoic acid may depolarize and cause the release of EAAs. Domoic acid may also depolarize the postsynaptic terminals, relieving the voltage-dependent Mg^{++} block at the NMDA-receptor. This action of domoic acid will allow a further depolarization by NMDA-receptor agonists released from the presynaptic terminals. Anomalously high blood levels of glutamic acid and aspartic acid, abundant in mussels[4,25,26], may likely occur as a result of gastrointestinal problems such as those reported[8]. Consequently, non-physiological amounts of aspartic acid and glutamic acid may reach the central nervous system and may reinforce the depolarization at the postsynaptic terminals. Such depolarization could become severe if ATP levels are low and the efficiency of the Na+/K+ pump is reduced[20], and may terminate in neuronal death.

In conclusion, cerebellar granule cells in primary culture are a valuable system for the understanding of the physiology and pathology related to neurotransmission by EAAs and for the evaluation of potentially neurotoxic synergisms between environmentally-related compounds, as reported here.

Mussels extracts have been prepared from non-toxic mussels and toxic mussels, by the following procedure: mussels were shucked, homogenized in a Sorvall Omnimixer at full speed setting, and the homogenate was lyophilized. For the determination of domoic acid, lyophilized mussels (245 mg) were suspended in distilled water (30 mL) and kept at 70°C for 10 min with stirring. After cooling at room temperature, the mixture was poured into absolute ethyl alcohol (60 mL) and then centrifuged in a bench top centrifuge. The clear supernatant was evaporated to about 15 ml in a rotatory evaporator at room temperature and the residue was adjusted to 25 mL in a volumetric flask. An aliquot of this solution was diluted 1:5 and used for the determination of domoic acid by HPLC, as previously described by Quilliam MA, Sim PG, McCulloch AW, McInnes AG. National Research Council of Canada. *Determination of domoic acid in shellfish tissue by high-performance liquid chromatography*. Atlantic Research Laboratory Technical Report 55, NRCC 29015. In brief, a Whatman Partisil ODS II, 10 μm column (25 cm x 4.6 mm) was eluted (1 mL/min) by aqueous 12.5% acetonitrile, adjusted to pH 2.5 with phosphoric acid

Schoefel variable wavelength detectors, set at 242 nm, and an HP 8452A diode array spectrophotometer, scanning from 200 to 300 nm every 5 s were used. Free amino acids were extracted by 0.1 N HC1, derivatized by OPA and measured by HPLC by A. Stehouwer, Research and Productivity Council, Fredericton, N.B., Canada, according to the method of Hill DW, Walters FH, Wilson TD, Stuart JD, Anal Chem 1979; 51, 1338-1341. The toxic mussels used in this study contained the following excitatory amino acids (in mg/g dry weight): Domoic acid = 2, Aspartic acid = 5, Glutamic acid = 2. In non-toxic mussels, domoic acid was

undetectable. For tissue culture experiments, 500 mg of lyophilized mussels were resuspended in 50 mL distilled water, heated at 70°C for 10 min, then centrifuged at 30,000 x g for 1 h at 40°C. Supernatant was lyophilized and the sample was then reconstituted with distilled water (6.4 mL). The pH of the solution was adjusted to 7.4. The final concentration of domoic acid, glutamic acid and aspartic acid in sample from toxic mussels was calculated from the values reported above.

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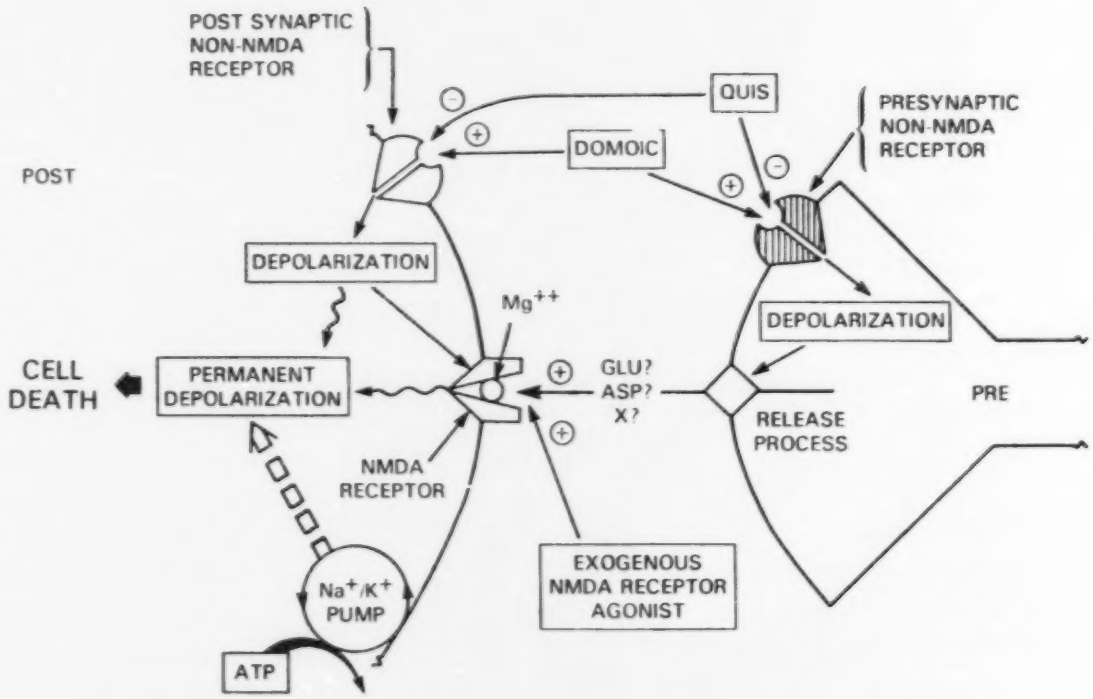
Figure 4
Hypothetical Scheme of Events Leading to Neurotoxicity by Excitatory Amino Acids†



†Domoic acid, present in toxic mussels, may reach the central nervous system and cause the release of endogenous excitatory amino acids from presynaptic terminals. Domoic acid may also act at postsynaptic terminals, facilitating the action of excitatory amino acids, either released from presynaptic terminals or occurring from exogenous sources (in this case aspartic acid and glutamic acid normally present in mussels). (Details in text).

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Discussion

Question from the floor: You mentioned that inhibition of the sodium pump potentiated the neurotoxicity of glutamate. Did this occur also for domoic acid?

A. Novelli: But the inhibition of the sodium potassium pump by low level of ATP is what we think is happening at the beginning for the relief of the magnesium block at the NMDA channel. So, a lower rate of sodium potassium pump may allow a slow depolarization in the cells relieving the magnesium block. Then glutamate starts to work. Of course, domoic acid doesn't need this first step. The poisoning of the sodium potassium pump by low level of ATP will affect the dramatic ability of the cells to survive

after any of these stimulations, glutamate and domoic acid. So yes, domoic acid action is affected and neurotoxicity is increased by any of these conditions.

Comment from the floor: I think this is an important point. If one is trying to explain triggering factors for example, the patients that are on conventional cardiac glycosides for arrhythmia treatment, and eating these mussels is going to be a real problem. Secondly, other agents which also inhibit the sodium pump profoundly, for example, vanadium would also be a potential candidate for this triggering factor.

G. Debonnel: You just mentioned the stimulation of GMP levels. You had an effect of MK-801 on kainic acid, but no effect on domoic acid. Could you explain that, is kainic acid acting on NMDA receptor?

A. Novelli: No, as I said the MK-801 effect on kainic acid stimulation was not dose dependent. I couldn't antagonize further kainic acid by increasing MK-801 up to I think 100 micromolar. Therefore, I think one possibility is that the domoic acid is releasing glutamate and the glutamate may act that way. But it can be some type of interference in the channel since we don't know if the channel is the same. There is speculation that the channel may be the same or similar, so, maybe, domoic acid is more efficient.

C. Pinksy: Did you say that the action of domoic acid is indifferent to the presence or absence of magnesium?

A. Novelli: Yes it is, because the absence of magnesium is actually reducing the effect of domoic acid instead of potentiating it as it happens for glutamate.

C. Pinksy: But why would you attribute that to the effects of magnesium on the enzyme when the kainic acid is affecting the same enzyme as domoic acid is.

A. Novelli: That is a very good question and I don't know the right explanation. But I can guess from some experience that I have working with GMP-cyclase. If you use the calcium ionophor, for example, you can induce the same stimulation on the GMP-cyclase. However, if you remove the magnesium from the extracellular medium, the stimulation that you can achieve is about half or even less than in the presence of magnesium. So, maybe the magnesium is needed for the activation on the cyclase and somehow is escaping from the cells. I don't know how, but ionophor is a natural shuttle for calcium and magnesium. With the domoic acid I don't know what is happening, but I guess that some magnesium is escaping from the inside to the outside where there is no magnesium.

18. Sensitive Murine Model and Putative Antidotes for Behaviorial Toxicosis from Contaminated Mussel Extracts

R. Bose, C. Pinsky, and G.B. Glavin

Abstract

The recent outbreak of "amnesic" mussel poisoning syndrome, attributed to domoic acid contamination of edible mussels claimed several lives and left many victims impaired with a peculiar loss of memory. We administered the whole mussel extract (WMX) and the mussel hepatopancreas extract (MHX, hepatopancreas being the major site for sequestration of domoic acid in mussels) in Swiss-Webster mice. A characteristic syndrome featuring sluggishness, scratching stereotypy, convulsions and death was noticed. Infant mice were some 3- to 4-fold more sensitive to the WMX toxicity. Kynurenic acid (KYN), an endogenous nonselective excitotoxin antagonist offered significant protection against the toxicosis after its onset had been provoked by the mussel extract. This observation emphasizes the feasibility of using KYN or related compounds in the therapy of poisoning from excitotoxins. As a logical extension of this possibility we examined the possibility that endogenous KYN could be exploited for similar protection against domoate toxicosis in our murine model. The time frame during which KYN was protective was increased by probenecid, a blocker of organic acid transport and by tryptophan, a precursor of endogenous KYN. We examined also the classical anticonvulsants phenytoin and ethosuximide, as well as dextromethorphan at its excitotoxin antagonistic dose.

The infant mouse model of domoate-toxicity holds promise for being developed into a rapid, sensitive, reliable and inexpensive biological assay for screening commercial batches of mussel for excitotoxin contamination. Kynurenic acid and dextromethorphan should be further examined as antidotes for possible therapeutic use in existing victims and in the treatment of future domoate toxicosis occurring here or elsewhere.

Introduction

The recent outbreak of domoic acid-contaminated mussel (*Mytilus edulis*) poisoning in eastern Canada [1,17,26] has left in its wake not only victims with persistent memory problems (see Presentation 3) and uncertain futures, but also the alarming possibility that it might happen again either at home or abroad. The lack of a suitable antidote against this unprecedented toxicosis is a matter of great concern as well.

Our laboratories have been concentrating on these 2 aspects and over the last few months, we have been developing a rapid, sensitive and reliable murine assay for detecting excitotoxin contamination in mussel extracts. We have also tested drugs which might serve as antidotes in this model of excitotoxin toxicosis. In earlier reports [16,27] we demonstrated significant protection by kynurenic acid against the gastrointestinal and neurological toxicities of domoic acid-contaminated mussel extract. Interestingly, this protective effect fell within a time window which permitted effective administration of the antidote after the onset of domoate toxicosis. In this present study we have examined the protective effect of endogenous kynurenic acid after pharmacological enhancement of its level in the brain. We have also examined the effect of reference anticonvulsant compounds and of dextromethorphan, another antagonist of excitotoxins.

Materials and Methods

Animals: Swiss-Webster mice (Charles River, adult males: 25 g mean body weight; 15-day-old suckling pups of both sexes: 5 g mean body weight) were housed in a light/temperature/humidity controlled vivarium with free access to food and water.

Mussel extracts

a) Whole mussel extract (WMX): Aqueous acidic extract of shucked whole mussels was prepared, at the Division of Biochemistry, Ottawa, Health and Welfare Canada, essentially in the same manner as prescribed for paralytic shellfish toxin [33]. This acidic suspension (pH 3.3 to 3.6) containing 0.15 mg domoic acid/mL was administered intraperitoneally (IP) into Swiss-Webster mice (adults or infants) at doses ranging from 5 to 40 mL/kg.

b) **Mussel hepatopancreas extract (MHX):** Hepatopancreas, 17.5 g, was dissected from toxic mussels and homogenized with 30 mL water in a Sorvall Omnimixer model 963c at speed setting "10" for 30 s. The homogenate was centrifuged at 3000 rpm, to yield, (with washings) a total of 60 mL of homogenate. An aliquot (1 mL) was used to determine the concentration of domoic acid, the remainder was freeze-dried. Domoic acid was determined by high performance liquid chromatography (HPLC), essentially as described by Bates et al. [2]. The freeze-dried MHX contained 8.5 mg domoic acid/g dry weight.

MHX was suspended in water for injection at concentrations ranging from 7.5 to 30 mg dry weight/mL. Final pH was measured to be 6.8 and injections given in volumes of 10 mL/kg, IP (estimated dose range of domoic acid being 0. 64 to 2.55 mg/kg).

Other pharmacological agents

All of the following solutions were injected in volumes of 10 mL/kg body weight.

Kynurenic acid: Kynurenic acid (KYN, Sigma) was dissolved in saline by adding 1 mM NAOH. The solution was neutralized using 1 mM HCl and saline was added to yield a final concentration of either 30 or 60 mg KYN/mL.

Probenecid: Probenecid (PROB, Sigma) was dissolved in saline using the identical procedures for alkalinization and neutralization as for KYN to yield a final concentration of either 10 or 20 mg PROB/mL.

l-Tryptophan: l-tryptophan (TRYP, Sigma) was dissolved in saline to yield a concentration of 10 mg/mL.

Phenytoin: Phenytoin (PHEN, Sigma) was dissolved in saline to yield a concentration of 1.5 mg/mL.

Ethosuximide: (ETSX, Parke-Davis) was dissolved in saline to yield a concentration of 20 mg/mL.

Dextromethorphan: (DEXM, Sigma) was dissolved in saline to yield a concentration of 2 mg/mL.

Experimental procedures

Dose-mortality response: Adult mice received either WMX at volumes of 5.3 to 60 mL/kg, IP, or BSA at 40 mL/kg, IP. Pups were given WMX at doses of 5 to 30 mL/kg, IP. Mortality was recorded over a period of 24 h. In another experiment, infant mice were injected with MHX at doses corresponding to 75 to 300 mg of dry powder (0.64 to 2.55 mg of domoic acid) per kg, IP. The incidence of scratching, convulsion and death were noted in these animals as were the latencies in min to the appearance of the first convulsive episode and of death. Survivors were continuously monitored for up to 4 h, based on earlier experience with WMX where all deaths occurred well within this period [7]. Interactions between the effects of WMX, MHX, KYN, PROB, TRYP, PHEN, ETSX and DEXM were also studied.

Behaviorial observation: Mice were observed for up to 4 h after being injected with WMX or MHX. Behaviorial signs of autonomic activity, motor activity, reactivity, stereotypes, convulsions (incidence and type), respiratory distress and motor incoordination were noted.

Statistical analysis: All data are presented either as percent incidence or as mean latency ± SE. Data were analyzed by ANOVA followed by Duncan's test for multiple comparisons. Incidence values were compared by X2 with Yate's correction.

Results

Behavior

The behaviorial signs observed were similar in adult and infant mice which received either WMX or MHX, these have been tabulated in Table 1. The scratching behavior was very characteristic and varied in intensity, generally increasing with time. It had a stereotypical seizure-like character and was usually followed by ataxia and clonic convulsions of extremities, often spreading to become more generalized. Occasionally, tonic convulsions were also observed. Many of the animals thus affected lapsed into a prolonged phase of "status epilepticus" with ongoing, low grade clonic convulsive activity almost always culminating in death of the animal. Just prior to death, mice showed evidence of respiratory embarrassment characterized by slow, irregular breathing, gasping and occasionally frank cyanosis.

Both WMX and HMX produced a steep dose-mortality response in all mice as summarized in Figure 1 and Table 2. Both incidence and latency to the appearance of the first convulsive episode were correlated with the dose of MHX, the incidence increasing from 20% to 100% and the latency decreasing from a mean value of 83 min to 18 min over a dose range of 75 to 300 mg/kg IP. There were no deaths in mice which received doses of MHX at 150 mg/kg or less. At higher doses the latency to death was negatively correlated with the dose of MHX.

Drug interaction experiments using mussel extracts

Experiment I (Table 2, Group IV and Table 3, Groups A and B): KYN pretreatment at 15 min before MHX gave no protection. However those mice which received PROB at 60 min and KYN at 15 min, both prior to MHX (200 mg/kg, IP) , showed lower incidence and significantly greater latency to the appearance of convulsions, with a marginally-reduced incidence of death as compared to the group which received MHX alone (Table 2, Group IV). Latency to death was longest in the PROB-KYN-MHX group followed by that in SAL-KYN-MHX group and the MHX group.

Experiment II (Table 2, Group V and Table 3, Groups E to G): Mice which received SAL, SAL, KYN and MHX (250 mg/kg, IP) were not significantly protected, although the latency to death in this group was 40% longer than that in the corresponding MHX treated mice (Table 2, Group V) . Mice which had received TRYP anti PROB before MHX (250 mg/kg, IP) were not protected as compared to the group that had received a similar dose of MHX alone. The group that had received TRYP, PROB, KYN and MHX showed a significantly prolonged latency to the appearance of convulsion and to death as compared to the group treated with MHX (250 mg/kg, IP).

Experiment III (Table 2, Group VI and Table 3, Groups C and D): With the order of KYN and MHX injection reversed, neither convulsions nor scratching were affected by the treatment, nor was the incidence of lethality. Latency to death in the KYN-treated groups, however, was greater than twice that in the mice which had received MHX (300 mg/kg, IP) alone.

The mice which were observed after PROB alone, or after TRYP followed by PROB, showed bouts of scratching which were less intense than those seen after MHX. They did not react to touch or pressure and showed signs of CNS depression such as stupor and loss of righting reflex. Their breathing was shallow but regular and they were cold to the touch; incandescent lamps were used to protect them from hypothermia.

Experiment IV (Table 4): When administered 20 min prior to WMX (7 mL/mouse, IP), both phenytoin and ethosuximide significantly prolonged latency to onset of convulsion and to death, without altering the incidence. However, when administered 60 min prior to WMX, phenytoin significantly prolonged the latency to onset of convulsion and death, whereas, ethosuximide gave no such protection.

In preliminary experiments, dextromethorphan administered at a dose of 20 mg/kg, IP 60 min prior to MHX given at 200 mg/kg, IP, protected significantly against death (Table 4) In other experiments where DEXM was administered up to 42 min after WMX, there was no protection against any of the toxic effects (data not shown).

Discussion

We have earlier reported [7] that the behaviorial profile of WMX was similar to that reported for neuroexcitatory amino acid (NEAA) analogues in mice [11,31] and in rats [18] with additional features indicative of central nervous system (CNS) depression in mice treated with WMX. An identical behaviorial profile was observed in mice which had received MHX. The different composition of the 2 extracts did not appear to affect the qualitative behaviorial responses, which presumably

can be primarily attributed to the domoic acid content of the extracts. We found infant mice to be 3- to 4-fold more vulnerable than adults to the WMX toxic effects. This age-dependent variation in sensitivity is not likely entirely due to the permeable neonatal blood-brain barrier, since domoic acid is lipid soluble [5]. An alternate explanation may reside in differential ontogeny of domoate and other NEAA receptor subtypes [3].

The behaviorial responses in Swiss-Webster pups after IP administration of MHX was very similar to that previously described [7] for infant and adult mice injected with domoic acid-contaminated WMX. Scratching was consistently observed in MHX-treated mice and was induced by the smallest dose of MHX used in this study, 75 mg/kg, IP. Such corresponds to the injection of 4.5 µg of domoic acid in 70 µL volume/7 g mouse pup, representing a dose of domoic acid at 0.64 mg/kg. At that dose scratching was observed in 80%, and convulsions in 20% of the animals. WMX contained approximately 0.15 mg of domoic acid/ml (representative mean value). The dose of WMX to induce scratching in 80% of infant mice was 6.9 mL/kg, corresponding to approximately 1.04 mg of domoic acid/kg. Quantitative differences in the equieffective amounts of domoic acid in MHX and WMX (0.64 and 1.04 mg/kg respectively) were possibly due to the different matrix of each, the modulatory components of which could be usefully explored.

It should be possible to exploit these observations and develop an infant mouse assay for facile detection of domoic acid contamination, utilizing scratching as the behaviorial parameter. Larger volumes (up to 300 µL IP/pup) of more dilute solutions of domoic acid or of contaminated mussel extract could be injected. A further increase in sensitivity could be achieved by selecting a lower incidence of scratching (e.g., 50%) as the response criterion. The lower limits of sensitivity would then approach 8 µg domoic acid/mL, yielding a 3- to 4-fold higher sensitivity than that reported recently for a behaviorial assay in adult CD1 female mice [10]. The infant mouse assay could thus provide a simple, rapid and robust method for large-scale detection of commercial batches of mussels and for screening of NEAA agonists.

Our earlier reported results [16,27] with WMX indicated that KYN, a nonselective antagonist at excitatory amino acid receptors [15], could protect adult mice from gastrointestinal and CNS toxicities (convulsions, death) only when administered after the domoic acid-contaminated extract. KYN was ineffective when administered 30 min prior to WMX but protected powerfully against WMX-induced convulsions when given between 30 to 90 min after WMX. It protected significantly (p) against WMX-induced lethality when administered between 45 to 90 min after WMX at a dose of 300 mg/kg IP. At a higher dose (600 mg/kg, IP), it offered effective protection (p) when administered at 28 min but not at 13 min after WMX [27]. Our present findings indicate a similar time-dependent protective effect of KYN against MHX toxicity in infant mice. Such a relationship of KYN protection to its tune of administration after the mussel toxin injection could reflect a chronologically-related activation of the receptive site influenced by domoic acid and the postulated associated strychnine-insensitive glycine site where KYN is thought to act primarily [19,20] in its antagonism of the NMDA subspecies of NEAA receptor stimulation. However, the extent to which a similar glycine site, or perhaps even use-dependent NEAA antagonism [34] is involved in the neurotoxicities provoked by domoic acid or by the extracts obtained from contaminated mussels remains to be investigated. Another factor influencing the relatively short duration of action and time-window dependence of KYN might be its rapid excretion from the brain [30] via organic acid transport mechanisms. Moroni et al. [24] have recently demonstrated that endogenous KYN can accumulate in brain after administration of PROB, a drug which blocks the cerebral egress of organic acids [25]. Accumulation of KYN, expected to occur after PROB in this present study, was likely further enhanced when TRYP, the natural precursor of endogenous KYN as well as of other biologically important substances [29] was administered.

The mice which received TRYP and PROB but not KYN fared little differently from the mice which received MHX alone, suggesting that endogenous KYN is insufficient to protect against exogenously administered mussel toxin. Combined treatment with TRYP and PROB, in the absence of exogenous KYN, might actually be detrimental because of the consequent increase in the accumulation of the endogenous excitotoxin quinolinic acid [23,29] which has been shown to be a convulsant [21] and to cause axon-sparing neuronal lesions similar to kainic and ibotenic acids [12,28].

We have observed scratching stereotypy in some of the mice injected with PROB alone. This was similar in character, though with much less intensity, to the scratching seen in mice after the administration of toxic mussel extracts [7], mussel hepatopancreas extracts (this study), domoic acid (this laboratory, unpublished) and other NEAAs [11]. Such PROB-provoked scratching could very well be due to cerebral accumulation of quinolinic acid. Although KYN has been shown to block quinolinic acid neurotoxicity [4,6,12,14,22] its levels in most mammalian brains are almost 100 times lower than those of quinolinic acid of corresponding species [24]. Moreover, El-Defrawy et al. [12] have shown that essentially an equimolar concentration of KYN is necessary to antagonize the neurotoxic effects of a given concentration of quinolinic acid.

Although endogenous KYN *per se* is not enough to protect against domoic acid-contaminated mussel toxicity, yet, the time-window of the protective efficacy of exogenously administered KYN can be expanded by TRYP and PROB or by PROB alone. It is not surprising that the protection we observed was not complete, being mostly a delay in the onset of convulsions or death, since the doses of MHX used here were relatively high. Future experiments might usefully employ lower doses of the toxic extracts to explore better the protective effects of the agents tested. While generally seen sequentially, convulsion and death seem likely due to toxicant effects on 2 or more distinct central systems. This is reflected in the more gradual dose-response relationship seen with convulsions as opposed to the rather abrupt and maximal appearance of death beyond a certain dose and by the rather steep decrease in time to death

with increasing dose of toxic extract. The manifest cause of death seemed to be due to cardiac irregularities as was evidenced by the extreme degree of slowing of the heart in animals dying of mussel toxicity. Implications of these phenomena are being further explored in our studies.

Both phenytoin and ethosuximide, 2 reference antiepileptics used in the treatment of grand mal and petit mal epilepsies respectively, were only marginally effective in delaying the onset of WMX-induced convulsions and death. In preliminary experiments, dextromethorphan, a commonly used antitussive which displays NMDA and glutamate antagonistic properties at high doses [8,9,13,32] protected significantly against HMX-induced death only when given prior to toxic mussel extracts.

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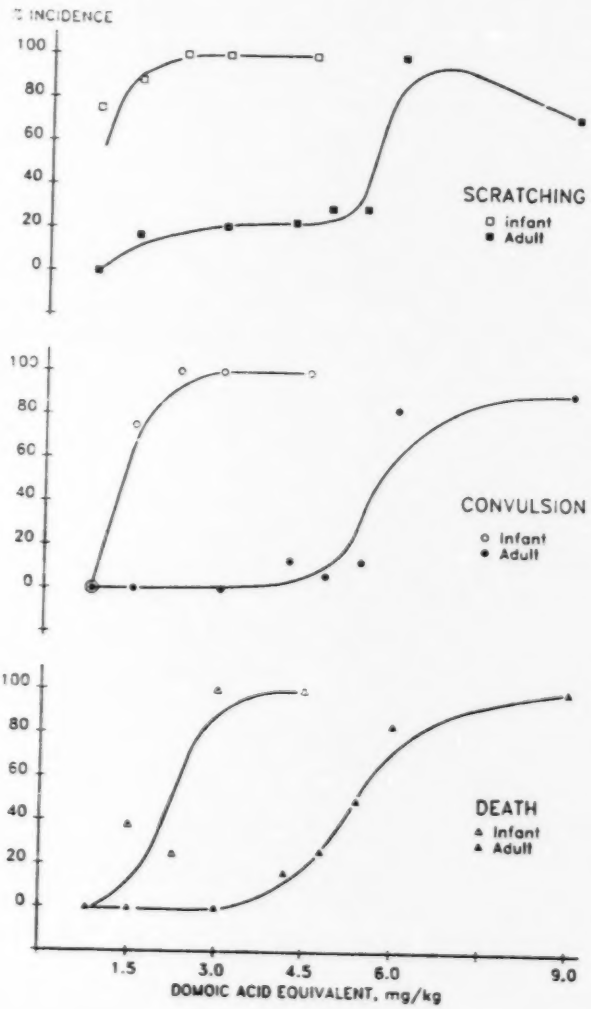
Figure 1
Differential Effects of WMX on Percent Incidence of Scratching, Convulsion and Death in Adult and Infant Swiss-Webster Mice



* doses of WMX have been presented as domoic acid equivalents
N = 6-19 (adults) and 4-6 (infants) for each dose-group

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Table 1.
Behavioral Profile in Swiss-Webster Mice
Following Administration of Domoic Acid-Contaminated Mussel Extracts

Frequently Observed Behaviors

- Sluggishness with maintained reactivity
- Scratching (body) stereotypy
- Clustered (heap) huddling
- Phalanx huddling
- Hind limb stretching
- Gait incoordination
- Clonic convulsion
- Respiratory distress, cyanosis
- Death

Infrequently Observed Behaviors

- Scratching (head) stereotypy
- Circling, barrel rolling
- Myoclonus
- Tonic convulsion
- Loss of righting reflex (post-ictal)
- Biting, hyperexcitability
- Prolonged, low-grade seizure ("Status Epilepticus")

Table 2.
Toxic Effects of Hepatopancreatic Extracts of Mussel Contaminated with Domoic Acid, on Swiss-Webster Mice

Group	Dose (mg/kg, IP)	Number	Scratching % incidence	Convulsions mean latency* (Percent incidence)	Death in min ± SE
I	75	5	80	83 [n=1] (20)	(0)
II	100	5	100	85 ± 3.5 (40)	(0)
III	150	5	100	50 ± 3.3 (80)	(0)
IV	200	7	100	33 ± 1.3 (86)	64 ± 6.9 (100)
V	250	3	100	33 ± 5.6 (100)	45 ± 1.0 (67)
VI	300	5	100	18 ± 0.5 (100)	30 ± 2.1 (100)

*after injection of extract

Table 3.
Effect of Kynurenic Acid, Probenecid and l-Tryptophan on Toxicity Produced by Hepatopancreatic Extracts of Domoic Acid-Contaminated Bivalves in Swiss-Webster Mice

Group	Injections #1	#2	#3	#4	Scratching % incidence	Convulsion mean latency* (Percent incidence)	Death in min ± SE
A	SAL	KYN[1]	HPX[2]	—	(83)	30 ± 2.4 (100)	74 ± 6.7 (100)
B	PROB[3]	KYN	HPX[2]	—	(100)	117 ± 0.5[a,b] (33)	92 ± 20.6 (83)
C	SAL	HPX[4]	KYN	—	(83)	21 ± 0.3[c] (100)	75 ± 8.2[e] (100)
D	PROB[5]	HPX[4]	KYN	—	(100)	16 ± 0.8[c,d] (100)	68 ± 6.0[e] (100)
E	SAL	SAL	KYN	HPX[6]	(100)	32 ± 3.6 (100)	63 ± 13.1 (60)
F	TRYP[7]	PROB[5]	SAL	HPX[6]	(100)	22 ± 1.0 (100)	40 ± 3.1 (100)
G	TRYP[7]	PROB[5]	KYN	HPX[6]	(20)	117 ± 7.5[f] (60)	136 ± 28.5[g] (100)

* latency measured from time extract injected (all given IP)
[1] KYN = kynurenic acid 600 mg/kg, IP given 15 min prior or post HPX
[2] Hepatopancreatic extract (HPX) 200 mg/kg
[3] Probenecid 200 mg/kg[2], 60 min before inj
[4] HPX = 300 mg/kg
[5] Probenecid 100 mg/kg, 60 min before HPX
[6] 250 mg/kg, IP
[7] l-Tryptophan 100 mg/kg, IP 3 h before HPX
[a] P≤0.01 when compared with Group IV Table 2
[b] P≤0.01 when compared with Group A
[c] P≤0.05 when compared with Group VI Table 2
[d] P≤0.01 when compared with Group C
[e] P≤0.01 when compared with Group VI Table 2
[f] P≤0.01 when compared with Groups V (Table 2), E and F
[g] (P≤0.05) when compared with Groups V (Table 2), E and F, G(P≤0.01)
n = 5 to 7 mice/group

Table 4.
Effect of Phenytoin, Ethosuximide and Dextromethorphan in Swiss Webster Mice Injected with Toxic Mussel Extracts

Injection 1	Interval (min)	Injection 2	Convulsion % Incidence	Convulsion Latency	Death % Incidence	Death Latency
Adult mice						
SAL (Control)	20	WMX	100	25±2.0	100	34±3.9
PHEN	20	WMX	100	39±2.7[a]	100	57±6.1[a]
ETSX	20	WMX	100	34±1.7[b]	78	52±4.2[a]
SAL (Control)	60	WMX	89	21±1.6	89	24±1.9
PHEN	60	WMX	89	36±4.3[a]	78	50±6.7[a]
ETSX	60	WMX	67	21±2.3	89	38±7.0
Infant mice						
SAL (Control)	60	MHX	100	28±0.6	100	79±29.6
DEXM	60	MHX	44	43±11.9	22[c]	54±2.0

n = 4 to 9 mice per treatment, all drugs administered IP
SAL = normal saline 10 mL/kg
WMX = whole mussel extract 40 mL/kg
PHEN = phenytoin 15 mg/kg
ETSX = ethosuximide 200 mg/kg IP
DEXM = dextromethorphan 20 mg/kg
[a] $P<0.01$
[b] $P<0.001_c$ compared with corresponding value in control group by ANOVA/Duncan
[c] $P<0.05$ compared by χ^2 with corresponding value in control group

Discussion

A. Tasker, Charlottetown: Dr. Bose as you know we have been working along very similar lines with regard to both developing a murine model, and I am pleased to see that your model is roughly equivalent in sensitivity to ours, and looking at a number of other aspects similar to what you have been doing and I wish I could share your enthusiasm for kynurenic acid. But I am afraid that in our hands we can find absolutely no evidence of an antagonistic effect with kynurenic acid at 200, 400 or 600 mg/kg, 30 minutes before, 15 before, same time, 15 after or 30 minutes after when compared to vehicle control either in saline or in conditions, such as you use pH 9 in water. I am wondering if you can offer any explanation for that discrepancy.

R. Bose: Well, in our studies we found that the therapeutic window actually started at about 45 minutes with a low dose of kynurenic acid and it went from 45 minutes to 90 minutes after we started administration. So if you stop at 30 minutes you might very well miss it. At a higher dose it was 28 minutes which is cutting it rather close. It can be 2 or 3 minutes longer or shorter depending on the experiment.

A. Tasker: Well, that may be true but that is only looking at high dose effects. Now we used 600 mg/kg kynurenic 30 minutes after and we record behaviours that can reliably detect 20 µg of domoic acid in mouse which is extremely low relative to the roughly 100 µg that you are looking at and still we see no protective effect. Now, I don't know whether maritime mice are different or what. The other question that I have concerns the use of exogenous tryptophan and I am just wondering how you can elude to the fact that this is an action exclusively on endogenous kynurenic acid because it is my understanding that exogenous tryptophan will do a number of things including elevating brain 5 HT levels. I am wondering if you have done any investigations to look at interactions between transmitter systems, such as serotonergic systems in response to the tryptophan loading. In other words, how do you conclude that it is an action on kynurenic acid?

R. Bose: We had thought about that and we would have proceeded along those lines if we had seen any protection with tryptophan. We didn't.

A. Tasker: But you did examine the possibility?

R. Bose: No, that was probenecid when it was combined with kynurenic acid exogenously. When probenecid-tryptophan was given before extract there was no protection. So we didn't pursue that.

A. Tasker: Then you did not investigate the possible role of 5 HT. Is that what you are saying?

R. Bose: No, we didn't, because we didn't see any protective effect of tryptophan for instance in this group where we gave tryptophan, probenecid, saline and extract. With no extra kynurenic acid there was no protection. So, probably with this kind of combination you'd expect that the 5 HT levels would increase but that didn't effect the parameters that we were studying. So we didn't pursue it further. Sure, it must be doing certain other things; for instance these animals were practically asleep. The type of convulsions was modified and there was hypothermia. That could be all because of the effect of serotonin. But as far as protection goes they didn't do anything.

C. Pinsky: I would like to comment on Dr. Tasker's question because, I am sure, we who did the experiments together in our group agree that is a very important question. I would like to point out that one thing I am quite sure of is it has nothing to do with the geographical distance between Winnipeg and Charlottetown but for sure has something to do with the time in which the kynurenic acid is measured. As Dr. Bose's data showed you, there is a very strict time-window and that actually has a clinical importance, because if there were a practical development of antidotes based on the pharmacological antagonists of excitatory amino acids, one would not expect someone who is going to dine on mussels to take an antidote before they go into the restaurant. The only time they would take the antidote is after they have had the warning signal of a gastric upset and that would be always after the administration and we noted quite happily with the kynurenic acid that it was more effective, almost exclusively effective, when given after the domoic acid preparations and if you give it before 30 minutes it simply has almost no effect. Now there are several possible explanations, some of which you have heard this morning, but just very recently it has been shown that kynurenic acid actually acts by several pathways other than direct competition at the neuroexcitatory amino acid receptor and there is a very good possibility that the 30 minute lag time we are seeing is due not only to blood-brain-barrier and kinetic considerations but to the dose dependent properties of the receptor and it should be a very interesting field and possibility to study. Thank you.

B. Stavric: One general comment — we heard that some phenomenon you observed with your extract with kynurenic acid was different than ours. Maybe it is important not only to measure the amount of domoic acid, when we are talking about whole extract, but what else is it in that extract. There is zinc, for instance, or magnesium, in the extract itself or other components. We don't specify what else is in that extract. Just one suggestion, it might be necessary to think what else might be in that extract which might have some effect one way or the other.

R. Bose: I think you have made a very important comment because we saw that even in the same extract, and it was refrigerated, and it wouldn't produce the same effect after say a 48-hour interval so that is why we had to go to the freeze dried extract which we prepared fresh for each experiment to keep it as consistent as possible, and certainly the composition of the extract might vary from one batch of mussels to the other. That is very important.

A. Tasker: I would just like to continue on that theme. That is an excellent point. We have also noticed some differences in terms of storage and things like that. That is why we traditionally do all of our studies looking at three different forms of domoic acid extracts from toxic mussels, where the domoic acid has been measured using HPLC, equivalent extracts from mussels taken from St. Mary's Bay prior to the appearance of domoic acid, and then subsequently spiked with domoic acid that's been purchased from a commercial supplier, and finally the domoic acid itself, the pure form, simply dissolved in water or saline as appropriate, I think that part of the reason for some

discrepancies in results that we heard throughout here may in fact be due to the different components of the extracts and I think it is important to control appropriately those conditions.

19. *Memory Loss Following Domoic Acid Intoxication from Ingestion of Toxic Mussels*

R.J. Zatorre

Disturbances in memory function may result from damage to any of several neural structures or systems. The literature in human as well as non-human amnesia indicates that bilateral limbic system destruction leads to the most severe and permanent form of memory loss, or amnesia, which is generally characterized by an inability to form new memory traces. Sometimes, this is accompanied by a certain degree of retrograde amnesia, or difficulty in retrieving information learned prior to the onset of the amnesia. In the most "pure" amnesic syndromes, the subject's other cognitive functions, such as language, reasoning, and so on, are generally completely intact[1]. Among the clearest and most intensively studied of such cases is H.M., who underwent bilateral excision of the amygdala and hippocampal region for relief of a seizure disorder. The result was an individual who is intellectually intact, but who is essentially unable to encode new information, including salient events in his own life. H.M., and other amnesic subjects, typically retain normal short-term retention abilities, so that as long as new information is kept in consciousness (e.g., by overt rehearsal), it is not lost, but as soon as attention is focused on another task then the new information will no longer be accessible.

The group of individuals who suffered domoic acid intoxication after consumption of Prince Edward Island mussels in 1987 present varying degrees of memory loss, and in some cases a true amnesia; we believe this is most likely due to damage to the hippocampal system from the excitotoxic action of domoic acid (Teitelbaum, Zatorre, Carpenter, Gjedde, Evans, Gendron and Cashman, unpublished observations). In this report the patients' neuropsychological profile will be described, and the evidence favoring the hypothesis of hippocampal destruction will be briefly mentioned.

A total of 14 patients were studied with an extensive neuropsychological battery of tests. These tests were administered between 4 months and 1 year following the onset of the acute illness. In most cases testing was spread out over several days. Of the original sample, 12 subjects showed some degree of memory loss; one subject showed a completely normal neuropsychological profile, while the other subject, at 87 the oldest in the group, was not testable due to global intellectual impairment. The data to be presented below, therefore, include only the 12 subjects of interest. It should be noted that premorbidly none of the subjects was known to suffer from any memory impairment, and this was confirmed by the families. Most subjects were very active in their occupations or in the community prior to eating the mussels.

The test battery included the Wechsler Adult Intelligence Scale-Revised[2] as an index of global intellectual ability. In order to measure memory function the

Table 1.
Results for 12 Subjects: WAIS-R IQ Ratings, and Scores for Tests of Naming and Comprehension (mean and range)

	Age	WAIS-R IQ Scores			Naming	Comprehension
		Verb	Perf	Full	(Max 26)	(Max 36)
Mean	64.8	109	96	104	20.7	32.8
Range	(44-84)			(81-130)	(17-24)	(30-36)

Table 2.
Results of Neuropsychological Tests: Memory

	Verbal Recall		Rey Complex Figure	
	Immediate	Delayed	Copy	Delayed Recall
Mean	12.8	7.0	25.3	4.13
Range	(6-20)	(2-17)	(17-30)	(0-10)
Normal	12-15	12+	25-36	12+

Note: Immediate verbal recall represents the sum of the words learned on the third trial of the Paired-Associates subtest and the mean of the number of units recalled from the Logical Memory (verbal passages) subtest of the Wechsler Memory Scale. Normal range estimated from normative data for appropriate age (Hulicka, 1966). Delayed verbal recall represents the sum of the words recalled from the Paired-Associate subtest after a delay plus the mean of the delayed recall of the two verbal passages. Visuospatial function is measured by the number of units of the Rey Complex Figure copied correctly, and visuospatial memory is assessed by the number of units recalled after a delay.

Wechsler Memory Scale was modified by the addition of a 90-minute delayed recall condition; visuospatial memory was assessed via delayed recall of the Rey Osterreith Complex Figure[3]. Language function was tested with an Object Naming test[4], and verbal comprehension was assessed with the Token test[5]. The Wisconsin Card Sorting Test, which is sensitive to impairments in concept formation often associated with frontal-lobe lesions[6] was also administered.

Table 1 gives the data for several of the non-memory tests. The average full-scale IQ of 104 reflects the generally normal and well-preserved intellectual status of most of the subjects. The range of scores (81 to 130) is correlated with each individual's premorbid estimated abilities; thus, the lowest score belongs to a patient who was a blue-collar worker, while the highest corresponds to a university professor. Many subjects obtained higher scores in the verbal scale than in the performance scale (which measures visuospatial and constructional ability). It remains unclear whether this simply reflects a characteristic of the sample, many of whom were highly educated, or if there may be some non-specific effect, such as generalized slowing, that affects the performance subtests preferentially. In any case, both verbal and performance IQs were in the average range.

Language testing did not reveal any abnormalities in any of the subjects, as shown by the scores on object naming and comprehension tests (Table 1). This finding is consistent with the good verbal IQ level mentioned above. Of the 11 subjects who received the Wisconsin Card Sorting Test, 8 passed the test and 3 did not, although 2 of these did succeed partially. This result suggests that concept-formation ability is generally adequate in this sample of subjects.

The principal abnormalities were observed on formal tests of memory function. The verbal memory score (Table 2) consists of 2 tests. In the first, the subject is read a prose passage and must repeat it immediately, and then a second passage is also presented; the subject's recall is scored for accuracy and number of ideas recalled. The second test included in the score involves learning a list of 10 paired-associate words, including some simple associations (e.g., east-west) as well as more difficult associations (e.g., crush-dark). After a delay of at least 90 minutes, but without warning to the subject, recall is once again elicited. This latter measure is generally much more sensitive to memory loss than immediate recall, since in immediate recall much information can be repeated from short-term memory.

Immediate verbal recall was not consistently impaired (Table 2), although some subjects had considerable difficulty even in this condition. A much clearer deficit emerged in delayed recall, in which the average score is clearly lower than normal, with some subjects' recall being essentially nil. In fact, in the case of 5 of the subjects, recall of any of the prose passages was impossible, even after prompting by the examiner about the content of the stories.
Paired-associate recall was also very poor, with some subjects obtaining the correct answer only on items with such strong associations that simple guessing would lead to the right answer.

The data for the Rey Complex Figure also indicate a severe memory disturbance. The score for copying the design was adequate in most cases, confirming the absence of any serious visuospatial or constructional deficit. However, when this design was to be recalled 45 minutes after it had been copied, none of the subjects performed well, and several of them were unable to draw even a single detail of the original design.

Informal questioning of the patients and their families also suggests some degree of retrograde amnesia in at least some cases, extending to months or years prior to the onset of the illness. For example, some subjects ware only vaguely aware about the events leading up to the consumption of the mussels; others are unable to recall clearly very salient events that happened prior to the intoxication (e.g., one patient could not recall the preparations for his daughter's upcoming wedding).

It is of interest that short-term retention seems to be intact in these patients, insofar as they were able to repeat a series of digits at a normal level immediately after presentation. They were also able to tap out a visuospatial sequence of blocks using Corsi's blocks. Thus, the memory deficit would appear to involve retention and/or retrieval of new information after it has passed out of consciousness.

Although several of the patients were elderly, this in itself does not account for the persistent memory troubles. First, as mentioned above, none of the subjects appeared to have any premorbid deficits. Second, the memory loss can be assessed with respect to age-corrected norms[7], and the deficits remain. Furthermore, there was no apparent correlation between degree of memory loss and age in our sample; indeed, the youngest patient, 44, a physics teacher with a doctoral degree, was among those who showed the most severe impairment in recalling verbal passages and the geometric figure.

In terms of the clinicopathological correlation, it is not possible to describe the precise locus of the lesion in any of the subjects studied neuropsychologically. Conversely, the subjects who died in the acute phase could not be tested for memory loss. However, the data from the neuropathological analysis are very suggestive, as all the autopsy cases revealed severe damage in the hippocampal region (Teitelbaum et al., unpublished observations). The neuropsychological features of the memory loss in the patients who survived are very similar to those of patients who have sustained known hippocampal damage bilaterally. Moreover, animal studies with kainic acid, an analogue of domoic acid, have shown that the hippocampal system is severely affected[8]. It is therefore entirely reasonable to conclude that the domoic acid contained in the toxic mussels led to varying degrees of damage to the hippocampus and related structures, which in turn led to the memory disturbance that is evident on neuropsychological testing.

At this point we are unable to explain the range of memory disturbance observed, with some subjects displaying a clinically evident amnesia, while others were only mildly impaired and were able to return to their original occupations. It is also not clear why one subject appeared to have no neuropsychological sequelae of toxic mussel consumption. It may be that some subjects consumed much more of the toxin than others, leading to varying degrees of deficit.

Another possibility is that some individuals may have eliminated or metabolized the toxin more quickly or efficiently than others, or there may have been different degrees of passage through the blood-brain barrier. These possibilities must remain conjectural for now, although it is notable that many of the most amnesic patients suffered from chronic illnesses, such as diabetes or renal failure, suggesting that these illnesses may have affected how the domoic acid interacted with the central nervous system.

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Discussion

M. Ball: I have a question with regard to the pathological follow up of two patients which Dr. Penfield saw during life, one of whom was operated on twice for intractable temporal lobe epilepsy. Gordon Matheson who did neuropathology at the Montreal Neurological Institute in those years published an important article in Archives of Neurology, I guess it was in 1974 or 1964, my memory slips me; but anyway in which it was shown that in the patient, who had been operated on twice, there was a potential correlation with the location or portion of the hippocampus that was resected surgically each time. Penfield hypothesized about lines of facilitation laid down in a stream of consciousness from the time of birth on. After the first surgical resection the patient had a four month retrograde amnesia and in a year or so later when he had to be operated on a second time they took out a little more of the hippocampus moving more posteriorly. He had a two year retrograde amnesia. Now we heard this morning that in the rats with the intraperitoneal kainic acid modeling, it seemed as though the histopathology was in the CA3 of the hippocampus rather than the caudal portion. If I could ask whether you or your colleagues, Dr. Carpenter or the others, have any evidence of the anteroposterior difference in the hippocampal damage of the ones who have already died.

R. Zatorre: Yes, well the question itself should probably be directed to Dr. Carpenter. I think the case that you described is the case of PB who indeed is quite illustrative and if I remember correctly what happened in his case was, you are quite right - the initial resection produced only a temporary problem from which he recovered and it did not cure his seizures. So, Penfield went in and did a more extensive removal and this did in fact cure the seizures but it did leave the man with a very severe amnesic syndrome and at the time it was not clear why from a unilateral excision there should be such a severe amnesic syndrome. In fact, when the autopsy was carried out, I think that was Matheson's paper, the interesting thing was that there was very considerable necrosis in the opposite hippocampal region. So, this is probably a subject who was functioning on one hippocampus and when you take it out you are left with very little to go on. But with respect to the retrograde amnesia aspect this is an interesting thing. We don't have any really good measures of retrograde memory which is why I did not present them. From the subjects descriptions it would appear that there is some extent of retrograde amnesia. Some of these patients are very vague when you ask them about their prior history. One of them, for instance, immigrated to Canada in the 1950s and he really doesn't remember very much about the circumstances related to his arrival in the country. So, there would seem to be some degree of retrograde amnesia but we have such poor measures of it that I don't think we can relate it to the extent of the anterograde component. With respect to the anterior and posterior hippocampus I don't know if Dr. Carpenter has a comment on that.

S. Carpenter: There seemed to be no difference between the anterior and the more posterior part of the hippocampus in these brains I looked at.

B. Pappas, Carleton University: Do you find that these people are extra sensitive to benzodiazepam which ordinarily will produce problems of memory and dementia-like syndrome in normal human beings? Do you find that these patients show a particular sensitivity to benzodiazepam?

R. Zatorre: I am not in the business of prescribing medications. I don't know if Dr. Teitelbaum has anything to say?

J. Teitelbaum: The patients as we are following them now, and there are 14, none of them are on benzodiazepam except one. That patient isn't in the study. He is very globally affected. He has been treated with Haldol^{R} almost since onset and it is not clear that his mussels came from P.E.I. the ones that he ate. The other ones are not on benzodiazepam, and haven't been. So I can't answer your question.

B. Pappas: The second question that I have relates to the observation that in amnesic patients, like HM, you could teach them a task like a mirror writing task and they will learn it at a relatively normal rate yet have no recollection of ever having encountered that experience before and this has lead to the distinction between procedural and episodic memory. I am just wondering if you have done similar things with these patients?

R. Zatorre: That is a very interesting point, I think. That is one of the reasons that we want to continue to study these patients because, I think, having established what they have they now allow us to test a number of hypotheses about the memory and one of them is

precisely this. Whether there is a sparing of implicit memory or procedural, whatever you want to call it and we are doing tests of that type with these subjects. We don't really have any data at the moment but we are certainly following that idea.

20. PET Studies of Domoic Acid Poisoning in Humans: Excitotoxic Destruction of Brain Glutamatergic Pathways, Revealed in Measurements of Glucose Metabolism by Positron Emission Tomography

A. Gjedde and A.C. Evans

Abstract

We used positron emission tomography to measure hippocampal and medial temporal lobe metabolism in brains of patients intoxicated by domoic acid from Prince Edward Island mussels. This analog of kainic acid specifically excites certain neurons in the hippocampus, and the study revealed a severe reduction of glucose metabolism in this part of the brain which paralleled the absence of long-, medium-, or short-term memory in these patients.

Introduction

In November 1987, 145 Canadians fell ill when they ingested mussels from Prince Edward Island that contained 100 to 300 mg domoic acid per serving (the number of patients may represent less than 1% of the number of people who actually ingested domoic acid in these amounts).

Of the recorded cases, approximately one third of the persons displayed neurological symptoms, 14 patients were selected for further study, 5 of these underwent positron tomographic measurements of regional glucose metabolism in brain, and 4 others died.

Domoic acid, a potent analog of kainic acid and the excitatory amino acid glutamate, is synthesized in several aquatic plants, including the seaweed *Chondria armata*. The affinity of domoic acid for the kainate-type glutamate receptor in the central nervous system is 3-fold higher than that of kainic acid. High-affinity kainate-type glutamate receptors are found in forebrain and on mossy fibre terminals in the CA3 layer of hippocampus. Originally, kainic acid was used to generate an animal model of temporal lobe epilepsy.

In the 14 studied cases of domoic acid ingestion, the investigation revealed that all patients suffered confusion and arousal deficits within 1 to 48 h of the ingestion. Maximal effects were apparent by 4 to 72 h, and improvement continued for 24 h to 12 weeks afterwards.

In 5 of the 14 patients studied at the Montreal Neurological Institute, focal motor, generalized tonic-clonic, and partial complex seizures developed. Four of the 5 had chewing movements of the mouth and myoclonus of the face, and all became comatose. These symptoms closely resembled those seen in animals suffering from kainic acid toxicity in which specific cells in the hippocampus are lost.

We formulated the hypothesis that the effects of domoic acid intoxication must mimic the effects of experimental kainic acid administration to animals, and that the consequences in humans must mimic the effects of ablation of the amygdala and hippocampus tissue in patients undergoing surgery for temporal lobe epilepsy.

To test this hypothesis, we determined the glucose metabolic rate of specific brain regions, including cerebral cortex, amygdala, and hippocampus, 2 to 3 months after the initial symptoms in 4 of the 14 patients.

Methods

Four patients afflicted by shellfish intoxication were studied in the McConnell Brain Imaging Centre of the Montreal Neurological Institute. Positron emission tomography (PET) studies and magnetic resonance imaging (MRI) studies were performed on all patients. Using procedures described by Evans et al.[1], matched MRI and PET planes were obtained to allow precise identification of regions of interest. The PET session was carried out between 2 and 4 months after the poisoning. Four normal, elderly subjects were scanned at equivalent position and orientation to provide comparable utilization values, particularly of the smaller limbic structures where parital volume effects would be most apparent.

MRI studies were carried out on Philips 1.5-T Gyroscan system using a slice width of 12 mm to match the axial resolution of the PET images. The PET measurements were made with the Therascan 3128, a 3-plane positron tomograph with a spatial resolution of 12 mm (transverse) by 11 mm (axial)[2]. Image reconstruction was performed with correction for random events, detector efficiency variation, attenuation, and scattered events. In each PET study, 3 horizontal planes parallel to the planum temporale and spaced at 12 mm intervals were scanned using a dynamic image acquisition schedule. At 45 min post-injection, 4 interleaved 3-plane scans were collected to yield a 12-plane data set finely sampled over the temporal area. Figure 1 shows the plane of scanning in the 4 patients in which the amygdala/ parahippocampal gyrus regions were most evident.

The regional glucose consumption (CMRGlc) was determined by IV injection of fluorine-18-labelled fluorodeoxyglucose (FDG). To improve the accuracy, the FDG studies were "dynamic," i.e., regional radioactivity in the brain was recorded repetitively during the study. The dynamic FDG studies yielded 18 records of tissue radioactivity in 20 min (10 1-min scans, 4 2-min scans, and 1 5-min scan) following intravenous FDG administration. Arterial blood was sampled for analysis of plasma radioactivity.

The positron tomograms were analyzed with the aid of customizable anatomical templates of known regions of the brain. The region-of-interest (ROI) templates were applied to the serial images obtained in dynamic studies. The use of the templates ensured reproducibility of the regional analysis across the patient data[1].

Figure 1
Normal Neuroanatomy of Plane of Scanning, Showing Location of Amygdaloid Nucleus and Parahippocampal Gyrus

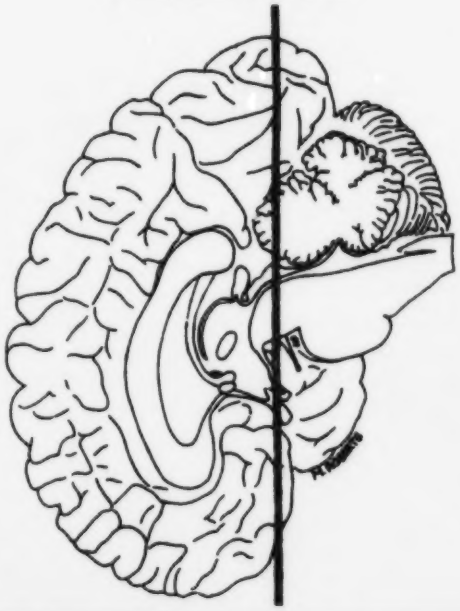
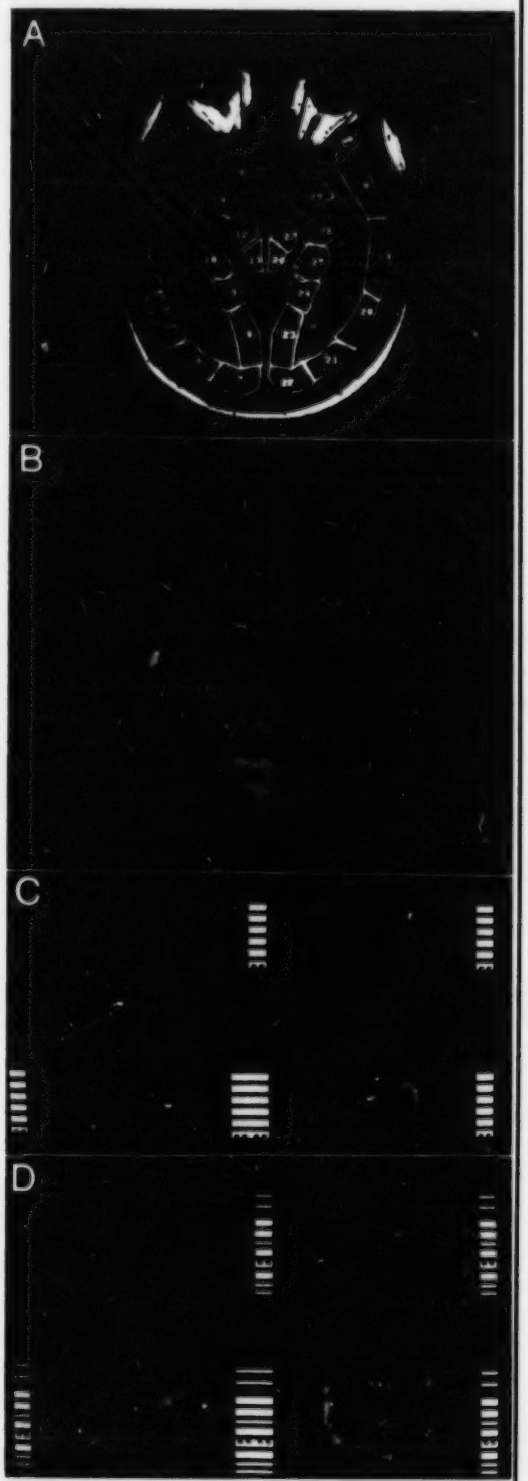


Table 1.
Average Glucose Metabolic Rate of Four Patients Suffering from Effects of Domoic Acid Poisoning and of Four Age- and Sex-Matched Controls

Age/Sex	Memory Score	IQ (Full-Scale)	CMRGlc	
			Cerebral Cortex	Amygdala/ Parahippocampal Gyrus
Year	(% of normal)	(% of normal)	(μmol hg^{-1} min^{-1})	
*69+5			22±3	19±2
46/F	129	116	40±2	26±1
59/M	92	117	33±1	26±1
84/M	55	86	26±1	15±1
84/M	17	92	11±0.4	3±3

* elderly control subjects (1F, 3M)

Figure 2



Panel A: T_1**-weighted MR-image of plane shown in Figure 1, overlaid with regional template adapted from stored atlas of patient suffering from domoic acid poisoning (59/M).**

Panel B: Glucose metabolism tomogram of same plane of same patient shown in Panel A.

Panel C: Quasi-3-dimensional reconstruction of glucose metabolism tomogram showing low glucose metabolic rates in parahippocampal gyrus in coronal plane marked A.

Panel D: 3-dimensional reconstruction of glucose metabolism tomogram of normal control subject, showing parahippocampal gyrus in coronal plane marked A.

The method used to calculate the metabolic rate for glucose in brain tissue *in vivo* was a modification of the method of Sokoloff et al.[3], formulated by Kuwabara et al.[4]. According to the Sokoloff method, the glucose metabolic rate is calculated as the net FDG clearance into brain at 45 min after injection, multiplied by the arterial plasma glucose concentration, and divided by the so-called "lumped" isotope correction constant. The value of the lumped constant normally used is that estimated by Huang et al.[5], at 0.42. In contrast, the modification of Kuwabara et al.[4], used constrained non-linear least-squares regression of time-activity records of brain tissue to obtain direct, individual estimates of the lumped constant and the glucose metabolic rate from the 18 radioactivity records of the first 20 min of PET data[6].

Results

The results obtained in 4 patients with varying degrees of severity of mussel intoxication are shown in Table 1. The glucose metabolic rate (CMRGlc) of cerebral cortex was also determined in 4 normal, elderly volunteers. Both cortical and amygdala-parahippocampal gyrus averages are listed in Table 1 relation to IQ and memory scores achieved by the patients.

Figure 2 illustrates the PET-MRI correlation on which the image analysis was based. Panel A of Figure 2 shows the T1-weighted magnetic resonance image at the level of the plane shown in Figure 1. Panel B of Figure 2 shows the functional glucose metabolism image of the same plane. Panel C of Figure 2 shows coronal reconstructions of a patient PET study (59/M). For comparison, Panel D of Figure 2 shows similar reconstructions of a study of a normal volunteer. Note the absence of the hippocampal formation in coronal slice A of Panel C when compared to coronal slice A of Panel D.

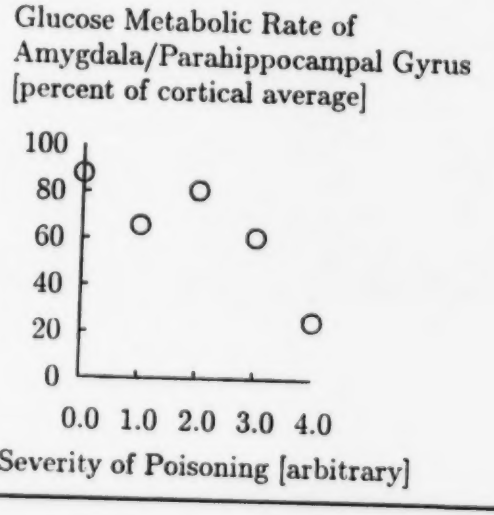
The relationship between the relative glucose metabolic rate of the amygdaloid nucleus/parahippocampal gyrus area, and the assessment of severity of the domoic acid poisoning of the 4 patients in which glucose metabolism was measured, is shown in Figure 3. The unit of the deficit score is arbitrary.

Discussion

The premorbid status of the patients listed in the table was fully functional. Only the 46-year-old woman recovered completely. All other patients sustained permanent memory loss that interfered with the resumption of work. The glucose metabolic rate of cerebral cortex revealed a roughly age-correlated decline. However, in addition to the general decline that may or may not have been accentuated by the toxic insult, the amygdala/parahippocampal gyrus regions sustained a further decline, relative to the cortical average. This decline is shown in Figure 3.

The study showed that the patients suffered a toxic insult which affected the functional activity of limbic forebrain structures, thus confirming the specific vulnerability of these structures to the pathological effects of excitatory amino acids. The specific memory defects of the most severely affected patients, in association with the focal decline of energy metabolism in the amygdala/parahippocampal gyrus area, confirms the participation of the structures in memory function.

Figure 3
Glucose Metabolic Rate of Amydaloid Nucleus and Parahipocampal Gyrus, Relative to Cortical Gray Matter Average, (Ordinate) Versus Arbitrary Patient Deficit Score (Abscissa)



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Discussion

Question from the floor: How you think the domoic acid gets into the brain? Does it use the existing dicarboxylate transporters?

A. Gjedde: If we take glutamate, it is a substance which teleologically and for other reasons shouldn't cross easily and doesn't seem to. These acidic amino acids have very poor transport though domoic acid and kainic acid, but I am sorry to say, have not been measured yet. This to me would be one of the first orders on the agenda in the future; to measure the blood-brain barrier permeability of these 2 compounds. But there is no such thing as complete impermeability of the blood-brain barrier. If the doses are sufficiently large or the exposure sufficiently long even proteins will get into brain tissue and I am sure that this is a simple mass effect of a large dose. It is not surprising to me that the effect is greater in rats than in monkeys and humans because of the holes in the blood-brain barrier. For instance, the circumventricular organs are comparatively bigger in smaller animals than they are in larger mammals which means that this what you might call, overflow effect in the rodents is much more important than it is in monkeys and in humans. In fact, a number of the discussions of peptides that were found in the rat brain were due to the fact that they spilled over from the circulation via the circumventricular organs and I am sure that that is the only way I can conceive these compounds get into the brain.

21. Gastrointestinal Effects of Contaminated Mussels and Putative Antidotes Thereof

G.B. Glavin, C. Pinsky, and R. Bose

Abstract

A recent outbreak of amnesic shellfish poisoning (ASP) in Atlantic Canada was characterized by severe gastrointestinal and central nervous system pathology. We examined the gastrointestinal effects of an acidic extract of blue mussels contaminated with domoic acid, the suspected toxin responsible for the ASP. We also tested the gastric effects pure domoic acid as well as a putative antagonist of neuroexcitant amino acid receptors, kynurenic acid.

Mussel extract produced gastric (antral) ulcers, duodenal ulcers, gastric and duodenal hyperemia and bleeding, as well as peritoneal ascites. Kynurenic acid protected significantly against extract-induced gastropathy, particularly when given 60 or 75 minutes after extract.

Pure domoic acid resulted in fatalities in all infant mice tested. These animals exhibited gastric bleeding and hemorrhage, especially at the higher doses employed.

In otherwise untreated rats, kynurenic acid exerted significant anti-stress ulcer and anti-gastric secretory effects, but was less effective at blocking ethanol-induced gastric lesions. We suggest that there may be both peripheral as well as central effects of kynurenic acid in modulating normal and pathological gastric function.

Introduction

In late 1987, an outbreak of atypical shellfish poisoning (cultured blue mussels, *Mytilus edulis* L.) occurred in Eastern Canada. Unlike paralytic shellfish poisoning, these patients were afflicted with severe and protracted central nervous system (CNS) disorders including seizures, loss of consciousness and memory disorders, some of which persist today. This syndrome has been called amnesic shellfish poisoning. In addition, the most seriously affected patients exhibited severe gastrointestinal distress, including nausea, vomiting and gastrointestinal bleeding. One patient who died, was found to have gastric and duodenal ulcers (Dr. J. Teitelbaum, Montreal Neurological Institute: personal communication, 1989). The agent implicated in these toxicities has been identified as domoic acid[1], an analog of the proconvulsant, kainic acid[2]. Among the most seriously affected surviving patients, the average amount of domoic acid eaten has been estimated to be 86 mg. The 2 most serious cases, both of whom exhibited gastrointestinal bleeding, consumed 214 and 218 mg respectively, or 3.0 and 3.3 mg/kg respectively (J. Teitelbaum, Montreal Neurological Institute: personal communication, 1989.)

We have examined the gastrointestinal effects of an acidic extract of poisonous mussels. Because of the reported stimulant effects of domoic acid on neuroexcitant amino acid receptors, we also tested whether kynurenic acid, a broad spectrum antagonist at such receptors[3] could obtund the adverse gastrointestinal consequences of toxin administration. In addition, we have characterized the gastrointestinal profile of kynurenic acid in healthy, non-toxin treated rats. Portions of this work have been noted in a previous brief report (see Presentation 18).

Materials and Methods

Animals: Male Swiss-Webster adult mice (Charles River; 25 g) male and female infant mice (15 days old; 5 to 10 g) and male Sprague-Dawley rats (University of Manitoba Central Animal Care Services, 200±10 g) used in these studies were maintained in a temperature and light-controlled (12:12 L/D cycle; lights on at 0700 h) room with food and water available *ad libitum*.

Drugs: Shellfish extract was provided by the Laboratory Centre for Disease Control, Health and Welfare Canada. Shellfish extract consisted of an acid extract of mussels, pH 3.6. Kynurenic acid was obtained from Sigma Chemical Co., St. Louis, MO.

Experiment 1

Adult mice were randomly assigned to groups and given single IP injections of mussel extract 0.5, 0.7, 0.8 or 1 mL of acidified BSA control, 1 mL[4]. The LD84 volume of extract was determined to be 1 mL per mouse[4], then used in subsequent experiments.

Mice dying as a result of extract treatment were immediately autopsied. The stomach and duodenum were excised and opened along the greater curvature and antimesenteric border, respectively. These tissues were rinsed with water, preserved in 10% formaldehyde and examined under a dissecting microscope with an ocular micrometer by an observer who was unaware of experimental treatments. The number and cumulative length (expressed in millimetres) of any gastric or duodenal ulcers were recorded as was the presence of hemorrhage, bleeding or hyperemia either in the stomach or the duodenum. The existence of peritoneal ascites was also noted. Mice surviving extract injection were killed by cervical dislocation and examined in the same manner.

By kynurenic acid studies, a suspension of kynurenic acid in 0.9% saline was given IP at a dose of 300 mg/kg in a volume of 0.25 ml at various times following extract treatment (at 45, 60, 75, 90 or twice at 15 and 30 min post-extract). All mice were examined as described above.

Experiment 2

Infant mice were given single IP injections of purified domoic acid (Atlantic Research Laboratories, National Research Council of Canada) at doses of 0.50, 0.84, 1, 4 or 8 mg/kg. Saline, at a comparable injection volume (0.05 to 0.10 mL), was used as a control. All mice died as a result of injection and were examined as described in Experiment 1.

Experiment 3

Cold-stress ulcer: Rats were deprived of food but not water for 24 h

prior to being immobilized in a cold (4 to 6°C) room for 3 h as described previously[5,6]. Immediately prior to stress, rats were randomly assigned to groups n=8 per group) and given a single intraperitoneal injection (1 mL volume) of saline, or kynurenic acid (Sigma) at doses of 100, 300, 600, or 1200 mg/kg. Following stress, all rats were decapitated, the stomachs examined as described in Experiments 1 and 2.

Ethanol ulcer: The method of Robert et al.[7], was used. Rats were deprived of food but not water for 24 h and then randomly assigned to groups (n=8 per group). Intraperitoneal injections of saline or kynurenic acid as described above were then given. All rats were then given a single PO injection of 1 mL of 100% ethanol (Canadian Industrial Alcohols, Corbyville, Ontario) by gavage needle. They were then returned to individual lab cages without food and water for 2 h. All rats were then decapitated and examined as described above.

Gastric acid secretion: Rats were first implanted with chronic indwelling gastric cannulas as described previously[8]. After a 14-day recovery period, gastric acid collection began. On each collection day, the cannulas were opened and the stomachs rinsed with 20 to 30 ml of distilled water to remove residual food. All cannulas were drained for 30 min following this rinse. A collecting tube and bottle were then attached to the cannula and all rats were then tested over 3 consecutive one-hour collection periods. The first hour consisted of a pre-injection baseline. At the beginning of the second hour, collection bottles were changed and injections of either saline, or kynurenic acid at the doses described above were given. At the beginning of the third hour, collection bottles were again changed and a post-injection collection period ensued. All rats were tested over 6 three-hour periods each separated by 96 h in the following order: saline, kynurenic acid 100 mg/kg, 300 mg/kg, 600 mg/kg, 1200 mg/kg, and saline again. Thus, each animal served as its own control, all injections were preceded and followed by a non-injection baseline hour, and all rats received a saline-only injection both before and after kynurenic acid.

Sample volumes were recorded and all samples were centrifuged at 2500 x g to remove residual debris. pH was determined and 1 mL aliquots titrated to pH 7 with a 0.01 M NaOH. Acid output was expressed as mEq/100 g body weight/h.

Results

Experiment 1

Table 1 summarizes the extract-induced gastric pathology data. Gastric (antral) ulcers occurred in mice given 1 mL of extract, while duodenal ulcers were observed in mice given 0.7 and 0.8 mL of extract. Massive peritoneal ascites occurred in 6 of 12 mice given 1 mL of extract, a significantly higher occurrence than seen in all other groups (Chi-square (4)=26.6; p<0.01).

Table 2 summarizes the effects of kynurenic acid on extract-induced gastric pathology. In mice given kynurenic acid at 60 or 75 min after extract, no evidence of either ulcers or ascites was detected. Only 1 incident of duodenal hyperemia occurred in the 75-min group. With kynurenic acid at 45, 90, or both 15 and 30 min after extract, gastric ulcers, ascites, and duodenal hyperemia were observed. Ascites incidence was significantly higher than BSA controls in both the 90 and the 15 and 30 min groups (Chi-square (5)=18.8; p<0.01).

Experiment 2

Table 3 summarizes the effects of pure domoic acid on gastric pathology in infant mice. Although no frank gastric or duodenal ulcers were observed, the highest doses of domoic acid used (4 and 8 mg/kg) produced a significantly higher incidence of gastrointestinal bleeding than seen in controls (p<0.05). No control mice and only one animal in the lower dose range (at 0.50 mg/kg) evinced gastric bleeding.

Experiment 3

Table 4 summarizes the cold-stress gastric lesion data. Kynurenic acid exerted a significant anti-ulcer effect (F(4,35)=4.92; p<0.01). All doses of

Table 1.
Effects of Shellfish Extract on Gastrointestinal Pathology in Mice (Mean ± SEM)

Group	Gastric Ulcer Incidence	No. Gastric Ulcers	Gastric Ulcer Length (mm)	Duodenal Ulcer Incidence	No. Duodenal Ulcers	Duodenal Ulcer Length (mm)	Incidence of Ascites
BSA control	0/12	0.0(0.0)	0.0(0.0)	0/12	0.0(0.0)	0.0(0.0)	0/12
Extract 0.5 mL	0/12	0.0(0.0)	0.0(0.0)	0/12	0.0(0.0)	0.0(0.0)	0/12
Extract 0.7 mL	0/10	0.0(0.0)	0.0(0.0)	1/10	0.1(0.01)	0.1(0.01)	0/10
Extract 0.8 mL	0/18	0.0(0.0)	0.0(0.0)	2/18	0.2(0.1)	0.4(0.1)	0/18
Extract 1.0 mL	3/12	0.5(0.1)	1.1(0.3)	0/10	0.0(0.0)	0.0(0.0)	6/12*

* significantly greater than all other groups (Chi-square; p<0.01)

kynurenic acid significantly reduced lesion severity (cumulative ulcer length) relative to saline-treated controls ($p<0.01$) while doses of 100, 300, and 600 mg/kg of kynurenic acid significantly attenuated the frequency of lesions appearing in the glandular stomach of stressed rats. Maximal protection against stress gastric damage occurred in the middle dose range (72%, 56%, and 68% reduction in ulcer length for 100, 300, and 600 mg/kg respectively).

Table 5 summarizes the ethanol gastric lesion data. Kynurenic acid again exerted a significant anti-ulcer effect ($F(4,35=3.98; p<0.04)$). These effects were less extensive than seen with stress gastric lesions. Specifically, only the doses of 600 and 1200 mg/kg of kynurenic acid significantly reduced ethanol lesion severity relative to controls ($p<0.05$ and 0.01, respectively). At a dose of 600 mg/kg, kynurenic acid also significantly reduced gastric lesion frequency relative to controls ($p<0.05$). The magnitude of the anti-ethanol lesion effects was less than that seen against stress lesions (6%, 5%, and 24% reduction in ethanol lesions for 100, 300, and 600 mg/kg respectively, versus 72%, 56%, and 68% reduction in stress lesions at the same doses, respectively).

Table 6 summarizes the effects of kynurenic acid on basal gastric acid output in conscious rats. Kynurenic acid significantly reduced basal gastric acid secretion ($F(5,35=6.65\ p<0.001)$. All doses of kynurenic acid reduced acid output in the hour immediately following injection, relative to pre-injection baselines ($p<0.01$). In addition, kynurenic acid at doses of 600 and 1200 mg/kg exhibited a sustained decrease in acid output persisting into the second hour following its injection ($p<0.01$).

Discussion

Health and Welfare Canada investigators have identified the potent neuroexcitant amino acid receptor agonist, domoic acid[9], as the major toxicant responsible for the 1987 outbreak in eastern Canada, of amnesic shellfish poisoning[1]. This syndrome was characterized by a constellation of severe gastrointestinal symptoms followed by protracted central nervous dysfunction. In Experiment 1, extract-induced unmistakable gastric (antral) ulcers, duodenal scarring and hyperemia, and peritoneal ascites. Kynurenic acid, a potent neuroexcitant amino acid receptor antagonist[3], given at various times after extract, blocked the adverse gastrointestinal effects. Comparisons between the data in Table 2 and those from the "Extract 1 mL" group in Table 1 indicate that kynurenic acid at all times post-extract, protected

Table 2.
Effects of Kynurenic Acid (300 mg/kg) on Shellfish Extract (1.0) mL-Induced Gastrointestinal Pathology in Mice (Mean± SEM)

Group	Gastric Ulcer Incidence	No. Ulcers	Ulcer Length (mm)	Incidence of Ascites	Incidence of Duodenal Hyperemia
BSA-90 min-KYN	0/10	0.0(0.0)	0.0(0.0)	0/10	0/10
Extract-90 min-KYN	2/15	0.1(0.1)	0.1(0.1)	3/15*	0/15
Extract-75 min-KYN	0/10	0.0(0.0)	0.0(0.0)	0/10	1/10
Extract-60 min-KYN	0/10	0.0(0.0)	0.0(0.0)	0/10	0/10
Extract-45 min-KYN	1/10	0.1(0.1)	0.2(0.1)	2/10	2/10
Extract-15 min-KYN 30 min-KYN	0/10	0.0(0.0)	0.0(0.0)	5/10*	0/10

* significantly greater than BSA-90 min-KYN (Chi-square; $p<0.01$)

Table 3.
Effects of Pure Domoic Acid on Gastrointestinal Bleeding in Infant Mice

Treatment	Incidence of GI Bleeding/Hemorrhage
Saline	0/4
DOM 0.50 mg/kg	1/4
DOM 0.84 mg/kg	0/4
DOM 1.00 mg/kg	0/4
DOM 4.00 mg/kg	3/4*
DOM 8.00 mg/kg	2/4*

* significantly greater than saline group (Chi-square; $p<0.05$)

significantly against extract-induced gastric ulcer incidence and length. This protection is consistent with the protection which kynurenic acid exerts against the neurotoxicities of the domoic acid contaminated mussel extracts in mice as presented by Bose at this Symposium (see Presentation 18).

The domoic acid *per se* is responsible for the gastric pathology observed in Experiment 1 is suggested by the results of Experiment 2, in which infant mice were given various doses of pure domoic acid. Infant mice were chosen for this work since worldwide supplies of pure domoic acid are very limited and very little of this compound was available to us. Unmistakable gastric hemorrhage and hyperemia were observed in most mice given higher doses of domoic acid. Frank ulcers were not observed, likely because most of these animals died shortly after injection, thereby preventing further degeneration of their gastrointestinal tract. These data, however, are remarkably consistent with the clinical observations of the humans who suffered the adverse gastrointestinal consequences of toxic mussel ingestion. We, therefore, have strong evidence that the mussel-induced toxicities observed in eastern Canada in late 1987 were due to domoic acid.

Kynurenic acid exhibited a strong anti-stress gastric lesion effect, a modest anti-ethanol lesion effect and a strong anti-gastric secretory effect in the rat. Ethanol lesions arise from a combination of massive luminal epithelial damage[10] and gastric hemostasis[11], while stress lesions are believed to involve gastric acid hypersecretion in addition to gastric motility aberrations[5,6,12]. It, therefore, appears that the greater efficacy of kynurenic acid against stress ulcer relative to ethanol ulcer is due to its anti-gastric secretory effects. This may also be due to a lack of involvement of prostaglandins in the anti-ulcer action of kynurenic acid in the gut. Unpublished observations suggest that prostaglandin synthetase inhibition by indomethacin pre-treatment does not affect the anti-gastric lesion effects of kynurenic acid in either the stress or ethanol models.

It is noteworthy that when given at doses of 500 and 1200 mg/kg, kynurenic acid markedly inhibited gastric acid output for at least 2 h after its injection. Since 3 h is the maximum duration for an open gastric cannula in the rat without dehydration of the animal, we have yet to determine the full duration of its anti-secretory effects. In addition, we are pursuing whether kynurenic acid is effective against stimulated acid output in rats (bethanechol, histamine and pentagastrin).

The question also arises as to the central versus peripheral location of these actions of kynurenic acid. It is well established that kynurenic acid is a broad spectrum antagonist at central excitatory amino acid receptors[13]. Whether such receptors exist in the periphery, specifically in the gut, remains to be determined, although preliminary reports suggest the existence of N-methyl-D-aspartate receptors in the guinea pig ileum[14]. Thus it is not unreasonable to postulate at least some peripheral sites of action for kynurenic acid in these experiments. In addition, because kynurenic acid is a non-selective neuroexcitant amino acid receptor antagonist, we cannot identify a specific receptor site of action at this time. However, we are currently examining more selective neuroexcitant amino acid receptor antagonists[15] with regard to their ability to block extract-induced toxicities.

Table 4.
Effects of Kynurenic Acid on Cold-Stress Ulcer Formation in Rats (Mean ± SEM)

Treatment	No. of Ulcers	Cumulative Ulcer Length (mm)
Saline	15.0 (1.0)	37.0 (1.0)
KYN - 100 mg/kg	8.0 (0.9)*	10.2 (1.7)**
KYN - 300 mg/kg	9.2 (0.9)*	16.4 (2.8)**
KYN - 600 mg/kg	6.4 (1.5)**	11.8 (1.9)**
KYN - 1200 mg/kg	15.0 (2.1)	20.3 (3.7)**

* significantly less than saline controls; $p<0.05$
** significantly less than saline controls; $p<0.01$

Table 5.
Effects of Kynurenic Acid on Ethanol-Induced Ulcers in Rats (Mean ± SEM)

Treatment	No. of Ulcers	Cumulative Ulcer Length (mm)
Saline	13.2 (1.9)	74.8 (13.1)
KYN - 100 mg/kg	14.5 (1.9)	70.3 (3.4)
KYN - 300 mg/kg	12.8 (2.2)	71.0 (12.9)
KYN - 600 mg/kg	9.4 (1.2)*	56.8 (4.4)*
KYN - 1200 mg/kg	10.0 (1.6)	38.6 (2.2)**

* significantly less than saline controls; $p<0.05$
** significantly less than saline controls; $p<0.01$

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Discussion

J. Olney: I think you should contact other Canadian colleagues — Pat and Edith MacGreer in Vancouver. When they were originally injecting kainic acid intracerebral ventricularly they found, I think, ulcers. They found bleeding through the urinary tract and they described some cardiac pathology. But I think it included all 3 of those organs and they hadn't done anything to the body. They were just injecting kainic acid directly into the brain.

G. Glavin: I will do that. It is not surprising that there would be some renal damage and possibly bleeding in the kidney but I'd have to check the details of that study with my colleagues in western Canada.

Table 6.
Effects of Kynurenic Acid on Basal (Non-Stimulated) Gastric Acid Secretion in Rats (mEq/100 g/h) (Mean± SEM)

Treatment	Hour 1	Hour 2	Hour 3
Saline	16.2 (0.8)	15.9 (0.3)	16.1 (0.6)
KYN - 100 mg/kg	14.9 (2.2)	11.8 (0.7)*	15.7 (1.1)
KYN - 300 mg/kg	17.8 (2.1)	7.3 (0.2)**	16.3 (1.8)
KYN - 600 mg/kg	16.5 (1.3)	5.2 (0.1)**	10.8 (1.0)*
KYN - 1200 mg/kg	16.0 (1.8)	5.1 (0.1)**	9.9 (0.8)*
Saline	15.7 (1.0)	16.3 (1.1)	16.5 (0.9)

* significantly less than hour 1; $p<0.05$
** significantly less than hour 1; $p<0.01$

22. *Panel Discussion: Treatment*

J.W. Olney, J. Teitelbaum, C. Pinsky, and G. Debonnel

J. Olney: I would like to open the discussion by pointing out that I think it is very important to try to assess what part convulsions play in the eventual pathology and clinical symptomatology both acute and chronic. If convulsions are largely responsible for much of the neuropathology and subsequent behaviorial clinical symptoms, then the treatment approach may be different than if the neuropathology is being produced by some other mechanism. If the neuropathology is essentially seizure-mediated pathology then the most effective mode of treatment — the primary mode of treatment — would be the most effective anticonvulsant that you can come up with. But if the neuropathology is occurring independently of seizures, then it is nice to prevent the neuropathology. So I would like to open up the discussion then in an effort to clarify that point first and I wonder if those of you who have been looking at domoic poisoning both in experimental animals and the human if you have been able to reach any tentative conclusion as to what role the seizures play in the eventual neuropathology.

J. Teitelbaum: I guess I could talk about the clinical aspects. A higher proportion of those patients who were most severely affected had seizures compared to those who were less affected. However, not all patients who were severely affected were found to have seizures. I think it would be important to mention, just from a clinician's point of view, that there were times when we didn't realize the patients were having seizures. Only the EEG showed diffuse abnormalities and in one case the debate was such that I was forced to bring a patient to the EEG room and give Valium^{R} to see if there'd be a change before we realized that the patient was having psychomotor seizures. So, it may play a more important role clinically then we realized at first.

J. Olney: I think that is a very important point. I was curious whether the clinicians dealing with these patients were aware of that. In experimental animals treated with kainic acid the seizure syndrome was relatively subtle and if you administer MK-801 you can prevent the behaviourial manifestations of the seizures, but if you sink depth electrodes into various regions of the brain the seizures are still going on. So, you can really be fooled whether or not there is substantial seizure activity going on in the brain by just a clinical observation. It is my impression that kainic acid neuropathology is almost entirely seizure-mediated and it wouldn't surprise me if domoic neuropathology is almost entirely seizure mediated and if that does turn out to be the case then there has to be an emphasis of finding the best anticonvulsant. You may be able to carry patients through the acute period with a good anticonvulsant preventing any convulsions until the domoic gets out of the system and then perhaps they will be spared brain damage.

C. Pinsky: We have been wondering about exactly the same thing ourselves and in that regard I have a slide that I brought with me. We just happened to do the experiment in time to make the slide. If I might show it perhaps it might be of some interest.

Here it is. In trying to discern differences between the lethality and convulsive toxicity of domoic acid in mice, and we use the mouse as our preparation of choice for several reasons which you have heard today. We decided to try recording EEGs from the relatively mildly restrained mouse and here you see an example of them. This permits us to perform experiments on the same animal, at present in a semi-chronic way but our plans are, of course, to keep the electrodes implanted chronically, so we can try and repeat experiments and watch the progression of neuropathology when we arrange our experiments to test this. What you see here in the upper tracing is a control recording. This is the EEG from a 10 g mouse and it is before any injection of the toxic extract. Please let me say that this mouse is being tranquilized by a small amount of diazepam. So, whether this is a normal mouse EEG or not, I don't know, and I don't know if anyone can define for you what is a normal mouse EEG. But, this is a mouse EEG in the presence of a just tranquilizing, not sedating, not anticonvulsant dose of diazepam. Three minutes later we injected the hepatopancreas extract of toxic mussels and within 10 minutes of that injection IP, we began to see spiking on the EEG and by 37 minutes it was quite steadily spiking. Now if one does no further intervention, that spiking will continue and become more frequent, the spikes will get larger and more frequent and I must emphasize that during all the time of this convulsive activity the mouse is absolutely immobile and looks as if it is sedated or sleeping — not even twitching, although there are some expressions of stiff tail and sometimes the tail vibrates but that is the only indication that the mouse is in any kind of excitement. Just to give another illustration of the action of kynurenic acid, this is kynurenic acid 300 mg per kg given to this mouse 47 minutes after the administration of the toxic extract and 20 minutes after that administration the EEG has begun to calm down. The spiking is much less frequent and much smaller and 2 minutes after that the spiking is almost all gone. This slide shows the kind the experiments we can do now with chronic or semi-chronic recording and we duplicate in this one experiment what took us in other circumstances large numbers of mice; we can do it with one mouse and repeat experiments on the table. Incidentally if one does not give the kynurenic acid, the spiking gets bigger and more frequent. I couldn't show it today, but we have experiments and details to show that, and I think it does emphasize that the kynurenic acid prevents seizure activity where diazepam does not. So, there seems to be considerable seizure activity that precedes the more toxic signs of gross overt toxicity that one would see that is the motor convulsions and the eventual death which we presume of course would be associated with neuropathology.

J. Olney: What dose of diazepam have you used?

C. Pinsky: In this case the operation is done and electrodes are implanted under

ether anaesthesia. As ether wears off which is fairly quickly we then give 10 mg per kg of diazepam, and occasionally during a long experiment — let's say after 4 hours — we have to top it up with another 5 mg per kg. But the 10 is just to be conservative. We get about the same result with 5 mg per kg and that much diazepam never prevents convulsions, even when it is given at the optimal time before the extract as Dr. Bose showed this morning. We needed a lot more to prevent convulsions.

J. Olney: In the rat treated with kainic acid, 25 mg per kg of diazepam has been used and it does prevent convulsions and the brain damage.

C. Pinsky: Is that at any particular time after the kainic acid or before?

J. Olney: It has usually been given before, but, in fact, we have done studies in which we want to allow kainic acid to damage the brain and then we want to stop the seizures at a certain point. We have been able to give diazepam 2 hours after seizuring, 3 hours, 4 hours, and we can stop it and allow the animals to live. Otherwise, they would most likely die eventually.

C. Pinsky: Well, there we see a very big difference between kainic acid and domoic acid. We can't stop a domoic acid seizure even with a high dose of diazepam given after the insult.

J. Olney: You can or cannot?

C. Pinsky: We cannot. It has to be given before pretreatment.

J. Olney: I think the other points that should be addressed have to do with the excitatory amino acid receptor types that might be targeted for therapeutic intervention, and again I would like to propose that although the domoic acid may be acting strictly at kainic acid receptors to trigger the neuropathological events, it is possible that N-methyl-aspartate receptors may come into play in one way or another. So, you might want to be targeting N-methyl-aspartate receptors at some point, but I am not sure whether any of that kind of approach is going to eventually be the primary therapeutic mode or whether one is going to be dealing with the convulsions as the primary target of therapy. Does anyone have any thoughts about this? Is Dr. Novelli here? Maybe he has some thoughts about the N-methylaspartate versus kainic acid receptors as therapeutic targets.

A. Novelli: I remember that sometime ago, Dr. Olney was thinking of the use of MK-801 to protect against neurotoxicity by glutamate in patients that were exposed to ischemic insult after the cardiac arrest. I would be very interested in seeing your paper in *Science* because of the effect that you anticipated in phencyclidine, if I am correct in remembering, protecting only to a certain extent and not completely. I think that at the moment it is not yet clear if it is possible to antagonize effects at the NMDA receptor very efficiently, even using drugs like MK-801 that looked so promising at the beginning.

J. Olney: I should probably show you some pictures of those changes that MK-801 and phencyclidine cause. I do have a couple of slides. This is a neuron here, there is one here, and here, and notice that there are some dark structures in the vacuoles. I think that those are mitochondria being incorporated within the vacuoles. The vacuoles are probably being formed from the endoplasmic reticulum. Now, this is 4 hours after MK-801 and the vacuoles are larger, and if you look throughout the cytoplasm of this nerve cell, it is hard to find any normal mitochondria. It looks like they have all been engulfed and are going through some kind of a process of dissolution. Remarkably, it looks like this kind of reaction is reversible. I say remarkably because I thought that if we could just keep administering MK-801 that we would cause the reaction to be progressive and eventually irreversible. But there is some kind of apparent tolerance mechanism in which the cell becomes tolerant to MK-801 and then turns off whatever the toxic mechanism is and you can just keep giving MK-801 and it doesn't occur any more. However, it is getting more and more complicated because we can produce the same effect with competitive NMDA antagonists and those compounds are acting at a totally different receptor. But the NMDA receptor where the competitive antagonists work, won't necessarily have the same tolerance mechanism built into it. So we haven't tested this yet but it is possible that that could be irreversible.

G. Debonnel (taking the Chair because Dr. Olney had to leave): In light of what Dr. Olney has said I think NMDA antagonist could be a good thing to have but it would be certainly a problem if you have this kind of lesion. I would have liked to ask Dr. Olney if he had the same kind of effect with ketamine which is also acting on the PCP 1 receptor and which is a substance already used by anesthesiologists so it could be readily available for this kind of patient. So, I really don't know if you would have the same kind of problem with ketamine, but maybe it could be an interesting way to block NMDA receptor without having this problem.

A. Novelli: If I can make a comment. It seems from what Dr. Olney said that a tonic activation of the NMDA receptor is needed for the normal function of the neuron. This is very interesting for me because from the cell culture point of view that seems to be the case also for the proper maintenance of the culture that I am using. They need a glutamatergic stimulation, very subliminal glutamatergic stimulation, in order to keep going up to 30, 40 days in culture. If you don't provide that the cells are dying much faster. So, I don't know if it correlates anyhow but I think it is an interesting speculation.

Comment from the floor: Just some comments from the whole animal research in ischemia apropos of your comment on ketamine. The results in ischemia, MK-801 and ketamine may provide a rough guideline although certainly in ischemia you have a different pathophysiologic situation and there seems to be a lot of noise in between many laboratories. MK-801 has been shown by some laboratories to be effective pre- and post-ischemia in varying animals and other laboratories have not been able to reproduce that. In my laboratory we found that MK-801 up to 20 minutes after ischemia is effective in preventing quantitated ischemic neuronecrosis. With regard to ketamine, you will remember that Dr. Olney showed us a slide where the potencies were ranked and ketamine is a much less potent noncompetitive antagonist than MK 801. We have found no effect with ketamine and most groups studying ketamine, there aren't as many as those studing MK-801, have found no effect with ketamine.

G. Debonnel: We presented last year at the Neuroscience Meeting results about interaction on the sigma receptors

and PCP 1 receptors. So if we can't use MK-801 for a toxicological reason, maybe we can just potentiate the effect of ketamine on the PCP 1 receptor via an action on the sigma receptor. With very low doses of Haldol[R], for example, we can potentiate about 10 times the effect of MK-801. We didn't try with ketamine but we have a fairly good chance to find what would be exactly the same effect.

A. Gjedde: I would like to say that the picture with MK-801 is perhaps starting to clear a bit because the effect of MK-801 seems to be in experimental focal ischemia. MK-801 given after the ischemic insult is capable of reducing the size of the infarct, whereas I think that the consensus is now that in global ischemia, anoxia, the effect is very controversial. In focal ischemia it binds only to the activated receptors which means that its almost a magic bullet focusing on the neurons in which the binding site has been exposed which is quite different from the effect you would see on normal neurons where there is no insult before the application of MK-801. I think it is very important to keep this distinction in mind and I think the only area where there is now incontrovertible proof of action is in focal ischemia.

Comment from the floor: Albert, I don't know if you consider bilateral carotid occlusion focal, in that it's only the forebrain, or global, in that it is both hemispheres, but we found that in bilateral carotid occlusion you certainly get an effect. With respect to the lesions Dr. Olney just showed I don't think we should focus on them as a serious impediment to therapeutic agents. These are small subcellular ultra structural lesions which are reversible and certainly you have much more severe lesions than that reversible such as dark neuronal transformation which we know occurs in aldehyde fixed tissue and is reversible so I don't think we should use that as precluding MK-801.

I. Hynie: I am just wondering whether it will be fair to say that only diazepam probably seems to have been of some value in the actual patients. As far as the future direction, this seems to be the matter of future research because, obviously, there is a lot of work to be done in vitro, on animals, and somewhere down the line we could develop applications for humans. This all probably would come under the heading of future research priorities which is basically the topic for the next panel discussion, but I'd like to ask: Is there something else other than Valium[R], which is already approved for use in human medicine, which can safely at this point be recommended as treatment if and when a patient appears who happens to be a known case of domoic acid poisoning. I suspect that the answer is no, but I would be very glad if somebody says that I am wrong.

G. Debonnel: I would just like to add that even if there are no clinical signs of seizures, I think it would be very important for all of these patients to have an EEG done to be sure that there are no seizures going on. Because, as Dr. Olney said, and as we have had the occasion to see on rats which we were given domoic acid intravenously under anaesthesia, they had absolutely no motor signs of seizures but when you are recording electrophysiologically in the CA3 region you can really see an epileptoid activity going on without any motor signs. So, I think it would be very important for all of the patients to have a regular EEG check to be sure there are no seizures going on.

J. Teitelbaum: There is even the additional problem that seizures are not always recordable on the EEG, especially if they are limbic which is going to be the case. It's the case of the experimental animal and sometimes the case of the human being and you don't always record this on the EEG, depending on where you have your electrodes whether you have sphenoidals or not. I think that for myself if a case should present now I would probably place them on a Valium drip even without seizure activity demonstrated. Even on the EEG if it were just abnormal with slow waves would be enough for me to start treating them.

C. Pinsky: I would have to concur with that from the theoretical point of view from what we have seen in our animal experiments but I do realize that there probably be the necessity of intensive care monitoring because I believe what we found was that you'd have to give the diazepam to levels close to respiratory depression which means you'd have to be prepared to assist respiration and certainly watch the patient continuously but that would be better than the alternative.

Comment from the floor: I guess we are all just agreeing with each other but again from the world of ischemia, we have given Valium[R] in ischemia and have found that it decreased the seizure incidence of post-ischemic seizures in the rat, improved the mortality rate and decreased the histological damage, even if the Valium is administered 20 minute post-ischemia. There was a clinical study by Kromholtz et al. in the *Journal of Neurology* where they studied the epileptic activity in patients that had global ischemia and found that those that had prolonged seizures did worse, not those that had a bit of seizures but prolonged seizures.

G. Debonnel: If I could add, I know that clinicians are sometimes afraid of high doses of benzodiazepam but I would like to remind them that there were several studies published for schizophrenic patients, for example, which we had treated with doses up to 400 mg of diazepam per day and they never had any respiratory problems.

A. Gjedde: One question. One of the effects of MK-801 in global ischemia has been noted to be a cooling of the brain and there have been several investigations showing that you can reduce glutamate release in models of ischemia by reducing body and brain temperature. Would it be something to consider in severe cases simply to cool the patient down?

J. Teitelbaum: It has been done in certain surgeries. It is not the first time it has been mentioned but it would certainly be complicated and even more difficult than placing them in the ICU for Valium[R] intravenously, but it could be done.

Comment from the floor: I think that definitive interpretation regarding the potential prophylaxis for this problem is actually quite tenuous. The data we have seen so far are predicated on 5 patients. Most patients are all suffering from various degrees of abnormalities apart from their age which is way above the normal. I think at this point what is needed is a rigorous examination of the basic data at the laboratory level and it is possible that some clues may emerge from this if the right questions are asked. I think the data presented by Dr. Novelli in his cerebellar neuron cultures are very intriguing. It was mentioned earlier this morning that there were considerable

discrepancies concerning the results using the muscle extracts. I would like to suggest that perhaps the chemist could get involved regarding the trace metal composition. For example, vanadium, copper, and zinc are very profound inhibitors of sodium potassium ATPase and other critical metabolic structures. What is the concentration of these 3 particular trace metals? Those are just a few suggestions that I would have at this point.

I. Hynie: Perhaps it would be fair to suggest that the past experience has been pretty well summarized here already and I believe since we have lost Dr. Olney, it may be better to publish the complete record of this discussion rather than to try to produce a brief summary. I am sure that the many theoretical suggestions for future research will be taken into account by the next panel on future research priorities.

23. Panel Discussion: Short-Term Research Priorities

F. Iverson, C. Pinsky, and J.L.C. Wright

F. Iverson: The late hour means that I have lost most of my original panelists. What I'd like to do is give a summary of research priorities that I saw come up during the rest of the talks and if anybody has a problem with it I will stop and we will discuss it. We have just dealt with a research priority and that is treatment. Certainly, whether we like it or not, we have almost fallen into the comfortable position of saying, "Yes it is domoic acid and a number of people are showing clinical signs, but what can we do about it?" It is a big problem. So, I think we have to agree that treatment is a priority. Carl do you have anything to say about that? I know you are doing research on that. We have covered most of it.

C. Pinsky: Well, in terms of priorities for research there were 2 that came up in my mind. One of them is, no matter what results do we have today, that we need a reliable source of domoic acid whose purity we know and whose potency we know. Dr. Gunner tells me that it is commercially available from a company in Canada now, which I think is a very salutary result of the epidemic, but I wonder what agency could look after the quality control. Can we rely on a company or can we expect and request quality control reports with each sample because in our laboratory, and many of our colleagues today have told us the same thing, we see differences between different batches of commercially available domoic acid and some of us are afraid of starting an experiment and investing a lot of money in enough domoic acid to do the experiment and then not being able to repeat it. So that is a very simple priority, but an important one. Now beyond that, another research priority that I see is the synthesis of new drugs, and the testing of existing drugs, that haven't been tested yet, because we didn't come up with very much when we asked what drugs are available. I think it is possible in practically every university. Everybody here, working in this field has a colleague who might be able to attempt the synthesis and we have wealth of knowledge available today concerning the neuroexcitatory amino acid receptors. We should be able to develop a drug which provides two things: 1) blockage of the toxic effects at certain receptors; 2) sufficient agonist effect to preserve the normal function of both synapses where we must have neuroexcitatory amino acids. That is what they are there for in the brain for some physiological function. Here I speak strictly as a pharmacologist. What I see we need is a partial agonist at those receptors which would normalize the function even in the presence of overstimulation or understimulation.

F. Iverson: Thank you very much. I think it is an important point. Certainly, we have not had that many different samples of domoic acid but I can say that one that we have had certainly wasn't up to snuff. And yes, there is a company in Canada.

J. Stewart: The company that is currently marketing domoic acid is Diagnostic Chemicals. They are located in the West Royalty Industrial Park, West Royalty, Prince Edward Island and they isolated from mussels that were harvested at the time of the outbreak. I gather that while they are aware that there has been some question of quality control that they are now much more rigorously testing the quality of their product and will provide people with information on request.

F. Iverson: I think yesterday we were also looking at defining the clinical syndrome and we didn't get any further and Ivo Hynie suggested that we put it down as a research priority. I think that is about all that we can do with that at the present time. I don't think we need anymore discussion about it and I just want to ask Dr. Teitelbaum if she has anything to add.

J. Teitelbaum: I have seen some of the attempts to define the clinical syndrome that looked quite good. I think we have to keep the gastrointestinal manifestations as part of it and emphasize the fact that some people will have only those manifestations. In terms of the neurological aspects acutely, probably the term "acute confusional state" is the best. Memory loss can't be put as part of an acute syndrome. You can't test it on a patient who is acutely confused. So, either use the points acute confusion changes in level of consciousness, seizures, myoclonus, and abnormal movements, or else group the whole thing as an acute confusional state. I vote for just putting those 3 categories down as the acute manifestations which can be followed or not by variable degrees of amnesia. That was discussed a little bit at different groups. The name is another problem.

F. Iverson: But generally you'd consider it pretty well a consensus with the people you have discussed with.

J. Teitelbaum: I think so.

F. Iverson: Do you have any other comment on that?

Question from the floor: We have tended to consider that problem in terms of what we now know in the analytical sphere. I would imagine within the next year or two there will be a lot of sensitive analytical methods available which would be available for use on serum and urine. Perhaps the presence of domoic acid would then become more of a criteria than it probably should be.

J. Teitelbaum: Oh yes, I was just defining a clinical syndrome, not mentioning the part of diagnosis or confirming the diagnosis with dosage. Right now that seems to be really difficult, but maybe stool samples would be helpful.

F. Iverson: Thank you. I think we heard this afternoon the necessity to continue follow-up on the patients, and to attempt to obtain a clinical-pathological correlation. Closing the gap into some of the animal studies, for instance, I think behaviorial studies seem to be an ideal situation that can be done as far as animal studies to try and relate to the human situation.

I. Hynie: I was just wondering since, I believe, Trish Perl and yourself met yesterday and put some of these things on paper. Would it be possible to drop me a note in writing, regarding what kind of definition of the syndrome you came up with?

J. Teitelbaum: Of course. I want also to address the next point about behaviorial studies. Dr. Zatorre isn't

here, but we have been working together on that aspect. We have organized to do these studies in the rat, inject them with domoic acid and then try to teach them mazes and other such tests of neuropsychological function and then take mice or rats who already know certain mazes who have learnt them and see what happens to their previous memory and their ability to learn new things. So, this is under way and we think this is a research priority.

Question from the floor: Could I add one comment to that. I think that in particular if we are going to move into behaviorial studies in rats we need to consider something which to me is one of the most confusing aspects, namely, the speed with which domoic acid passes across biological membranes, and there are 2 areas where I think it needs to be defined. One which was also mentioned today, the speed with which domoic acid can pass across placental membranes to affect the fetus is one problem which can also be addressed in animals and the other is the speed at which it passes the blood-brain-barrier. In both cases I think it will be difficult to make behaviorial studies exact if they are based only on measurements of domoic acid that are measured in the blood stream and/or dosage if we do not know the speed at which it passes into the brain. I think that is a measurable entity. I think it can be done rapidly and accurately if there is a reliable source of labelled domoic acid. I think we could put together a team in Montreal that would be very happy to examine that issue and that is why I would like to urge that in addition to the considerations of getting a reliable source of cold domoic acid that we consider getting a reliable source of hot domoic acid as well. Possibly in view of the monkey experiments that have been performed, perhaps also devise ways in which there could be support for the labelling of domoic acid for PET studies. There are 3 or 4 PET centres in Canada and I think there should be made an effort to label domoic acid with positron emission tomography in monkeys, perhaps in the monkey colonies that exist in Ottawa. I think that is an immediate priority. I think that none of these studies that will be performed in the future based on dosage or simple blood concentration will be worth much without knowing what that means in terms of the entering to the brain.

F. Iverson: Thank you very much. I can see how everybody is aiming at my list here so I might as well just read them all off. These priorities, I think, are important and I gather you do as well. First of all, analytically we need a method, that would give us sub-ppm levels to be used. For blood and tissue distribution studies. We would like to know something about metabolism. Certainly tissue distribution or metabolism studies would go along much faster, much better if we had a good source of labelled material, as you mention. Certainly you can get tritium labelled material made for you, but carbon 14 in our case would be much better.

Question from the floor: Also C13 or carbon C11-labelled domoic acid.

F. Iverson: We all have our specialties, and certainly I think for other pharmacological studies as well. We plan to go ahead and do what is basically another teratogenicity study but with larger amount of material. I think we are all aware that radiolabelled material and/or a better method will help us address this problem of the uptake. This transfer across the gut — the absorption phenomenon that seems to be a big problem with many of the studies and this can be addressed with better methods and finally, back to the behaviorial studies again. I think that in as far as looking after the mussels and ensuring that they don't get on the market, I think we seem to be doing a very good job and the people involved should be congratulated.

Question from the floor: I was just going to suggest that along in your behaviorial studies and/or physiological studies it might be interesting to include senescent animals in view of the fact that most of the patients were quite old, and also possibly to include animals that have various types of syndromes that were seen in these people since there are animal models for a number of those, such as diabetes and hypertension, etc.

F. Iverson: That is certainly a consideration. If possible it could make things a little difficult for some people, but that is certainly a consideration.

Question from the floor: I have one simple point. It might be valuable from the point of view of labelled domoic acid then to find out how good an indicator kainic acid is of domoic acid because it has been available for a long time. It can be bought in a labelled form. To what extent can we use kainic acid instead of domoic acid in some of this research? That should be established as well.

F. Iverson: Any other comments on that?

Question from the floor: Although it is tempting to follow the kainic acid model because of the similarities we should never forget that dissimilarities exist. I think we ought to understand first domoic acid a little bit better before we jump on the kainic acid.

C. Pinsky: I would like to make a specific comment on that particularly from the observations of Zachek and Coyle who have been studying this matter very intensively. They have made the point that domoic acid is very different from kainic acid and in fact from all the other neuroexcitatory amino acids, in that for a given amount of epileptic activity generated by a given dose of the drug there will be much more neurotoxicity from domoic acid than from any of the other neuroexcitant amino acids. So, I think from the point of view of the salient events that we are looking at it might be better to concentrate on domoic acid. We just couldn't extrapolate from one drug to the other.

F. Iverson: Thank you very much, Carl, for the warning. Other comments, research priorities?

J. Campbell: Just in following up on a point that was made about senescent animals, are there any particular caveats about species here? I bring this up only because I have been told, we have a fair number of senescent cynomolgus monkeys in our own shop. Do they have any particular promise that might relate to the kind of priorities you are talking about?

F. Iverson: Well, certainly they may have. That is why I said it might give people some problems. It just depends on what species you are going to use. If we are lucky enough to have senescent monkeys it might be worth considering, and other people would consider senescent rats. I guess it is the time-frame you are looking at.

Question from the floor: I think it would be extremely important to establish here the blood-brain-barrier

permeability in these older monkeys. We know that it has been a theory that human dementia may have a component from the blood-brain barrier breaking down due to age. I think it would be very interesting to use labelled domoic acid to see whether the permeability of the old monkey blood-brain barrier to domoic acid say is different from that of the younger monkey. That is a study that I would immediately fund if I had any money.

F. Iverson: Other comments?

Comment from the floor: I guess this research priority has been stated in different words, but just to state it concisely I think one of the priorities would be to see what is effective in preventing the neuropathology and long term behaviorial consequences. What is the best route of administration and how late after the onset of the domoic acid ingestion or administration is this effective.

F. Iverson: I agree. I hope that one will certainly come out of any studies we want to do.

Question from the floor: I really do hope that just like with an NMDA receptor, in future we'll have a much more selective and efficient antagonist of the kainic receptor or either of the kainic subtypes of receptors because we could find that, in fact, there is not one type but different subtypes of receptors. So, we definitely need in a very urgent way several kind of selective antagonists for this subtypes of receptors to be able to do these sort of studies.

F. Iverson: If there are no more questions maybe I'll just very briefly go over what I perceive to be the major priorities raised in this discussion:

1. Identify a source of supply for domoic acid to be used in additional investigations. Domoic acid is available from:
   Diagnostic Chemicals
   West Royalty Industrial Park
   Charlottetown
   Prince Edward Island
   C1E 1B0.
2. Preparation of Radiolabelled domoic acid. Carbon 14 (14C) labelled domoic acid would facilitate required studies on the absorption, distribution metabolism, and excretion of domoic acid. Tritium (^{3}H) labelled domoic acid can be obtained from Amersham and would be suitable for receptor studies.
3. Development of an analytical method suitable for determination of domoic acid in tissue (blood) at levels below 1 ppm. Current methods are suitable for monitoring purposes but lack the sensitivity required for tissue distribution studies. The methods would also be required for confirmation of domoic acid ingestion.
4. Conduct neurobehavioral studies. Studies based on visual and auditory response will help define the neurotoxicity of domoic acid.
5. Complete ongoing work relating to receptor affinity. Species differences in receptor affinity may impact on the neurotoxicity of domoic acid.
6. Continue studies designed to establish the effectiveness of antidotes (antagonists) and other treatment procedures applicable to individuals exhibiting domoic acid intoxication.
7. Complete teratogenicity studies. Negative preliminary studies should be confirmed with a more extensive study.
8. Conduct a subacute oral study in a suitable animal model. A repetitive oral dose study should be conducted when sufficient domoic acid is available. This study will define the potential of domoic acid to produce effects in a more chronic, low dose situation.
9. Complete the definition of the clinical syndrome used to define patients afflicted with mussels associated intoxication.
10. Continue the follow-up of patients.
11. Complete studies to define specific conditions that may influence toxicity, e.g., blood-brain barrier permeability, absorption, renal damage, and gastrointestinal damage.